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(54) **Non-invasive Detection of the Integration of a Transgene in Mammals**

Nicht-invasive Feststellung der Integration eines Transgens bei Säugetieren

Detection non-invasive de l'integration d'un transgène dans les mammifères

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(73) Proprietor: **THE BOARD OF TRUSTEES OF
THE LELAND STANFORD JUNIOR UNIVERSITY**
Stanford, CA 94304 (US)

(72) Inventors:
• **CONTAG, Christopher, H.**
San Jose, CA 95129 (US)
• **CONTAG, Pamela, R.**
San Jose, CA 95129 (US)
• **BENARON, David, A.**
Redwood City, CA 94062 (US)

(74) Representative: **VOSSIUS & PARTNER**
Postfach 86 07 67
81634 München (DE)

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Description

[0001] The present invention relates to noninvasive methods and compositions for detecting, localizing and tracking light-emitting entities and biological events in a mammalian subject.

[0002] The ability to monitor the progression of infectious diseases is limited by the current *ex vivo* methods of detecting and quantifying infectious agents in tissues. The replication of an infectious agent in a host often involves primary, secondary and tertiary sites of replication. The sites of replication and the course that an infectious agent follows through these sites is determined by the route of inoculation, factors encoded by the host as well as determinants of the infecting agent.

[0003] Experience may offer, in some cases, an estimate of probable sites of replication and the progress of an infection. It is more often the case, however, that the sites of infection, and the pace of the disease are either not known or can only roughly be estimated. Moreover, the progression of an infectious disease, even in inbred strains of mice, is often individualized, and serial, *ex vivo* analyses of many infected animals need to be conducted to determine, on the average, what course a disease will follow in an experimentally infected host.

[0004] Accordingly, it would be desirable to have a means of tracking the progression of infection in an animal model. Ideally, the tracking could be done non-invasively, such that a single animal could be evaluated as often as necessary without detrimental effects. Methods and compositions of the present invention provide a non-invasive approach to detect, localize and track a pathogen, as well as other entities, in a living host, such as a mammal.

[0005] The invention includes a noninvasive method for detecting the localization of a biocompatible entity in a mammalian subject. The entity can be a molecule, macromolecule, cell, microorganism (including a pathogen), a particle, or the like.

[0006] The method includes administering to the subject a conjugate of the entity and a light-generating moiety. Light-generating moieties are typically molecules or macromolecules that give off light. They may generate light as a result of radiation absorption (e.g. fluorescent or phosphorescent molecules), or as a result of a chemical reaction (e.g. bioluminescent proteins). Exemplary light-generating moieties are bioluminescent proteins, such as luciferase and aequorin, and colored or fluorescent proteins, such as yellow fluorescent protein and ferredoxin IV.

[0007] The moiety may be conjugated to the entity by a variety of techniques, including incorporation during synthesis of the entity (e.g. chemical or genetic, such a fusion protein of an antibody fragment and a light-generating protein), chemical coupling post-synthesis, non-covalent association (e.g. encapsulation by liposomes), *in-situ* synthesis in the entity (e.g. expression of a heterologous bioluminescent protein in a transformed cell), or *in situ* activatable promoter-controlled expression of a bioluminescent protein in cells of a transgenic animal stimulated by a promoter inducer (e.g. interferon-activated promoter stimulated by infection with a virus).

[0008] After a period of time in which the conjugate can localize in the subject, the subject is immobilized within the detection field of a photodetector device for a period of time effective to measure a sufficient amount of photon emission (with the photodetector device) to construct an image. An exemplary photodetector device is an intensified charge-coupled device (ICCD) camera coupled to an image processor. If the image can be constructed in a time short relative to the time scale at which an "unimmobilized" subject moves, the subject is inherently "immobilized" during imaging and no special immobilization precautions are required. An image from the photon emission data is then constructed.

[0009] The method described above can be used to track the localization of the entity in the subject over time, by repeating the imaging steps at selected intervals and constructing images corresponding to each of those intervals.

[0010] The method described above can be used in a number of specific applications, by attaching, conjugating or incorporating targeting moieties onto the entity. The targeting moiety may be an inherent property of the entity (e.g. antibody or antibody fragment), or it may be conjugated to, attached to, or incorporated in the entity (e.g. liposomes containing antibodies). Examples of targeting moieties include antibodies, antibody fragments, enzyme inhibitors, receptor-binding molecules, various toxins and the like. Targets of the targeting moiety may include sites of inflammation, infection, thrombotic plaques and tumor cells. Markers distinguishing these targets, suitable for recognition by targeting moieties, are well known.

[0011] Further, the method may be used to detect and localize sites of infection by a pathogen in an animal model, using the pathogen (e.g. *Salmonella*) conjugated to a light-generating moiety as the entity.

[0012] The invention may find application in a noninvasive method for detecting the level of a biocompatible entity in a mammalian subject over time. The method is similar to methods described above, but is designed to detect changes in the level of the entity in the subject over time, without necessarily localizing the entity in the form of an image. This method is particularly useful for monitoring the effects of a therapeutic substance, such an antibiotic, on the levels of an entity, such as a light-emitting bacterium, over time.

[0013] In one embodiment, the invention includes a noninvasive method for detecting the integration of a transgene in a mammalian subject. The method includes administering to the subject, a vector construct effective to integrate a transgene into mammalian cells. Such constructs are well known in the art. In addition to the elements necessary to integrate effectively, the construct contains a transgene (e.g. a therapeutic gene), and a gene encoding a light-gener-

ating protein under the control of a selected activatable promoter. After a period of time in which the construct can achieve integration, the promoter is activated. For example, if an interferon promoter is used, a poly-inosine and -cytosine duplex (poly-IC) can be locally administered (e.g. footpad injection) to stimulate interferon production. The subject is then placed within the detection field of a photodetector device, such as an individual wearing light-intensifying "night vision" goggles, and the level of photon emission is measured, or evaluated. If the level is above background (i.e. if light can be preferentially detected in the "activated" region), the subject is scored as having integrated the transgene.

[0014] In a related embodiment, the invention includes a noninvasive method for detecting the localization of a promoter-induction event in an animal made transgenic or chimeric for a construct including a gene encoding a light-generating protein under the control of an inducible promoter. Promoter induction events include the administration of a substance which directly activates the promoter, the administration of a substance which stimulates production of an endogenous promoter activator (e.g. stimulation of interferon production by RNA virus infection), the imposition of conditions resulting in the production of an endogenous promoter activator (e.g. heat shock or stress), and the like. The event is triggered, and the animal is imaged as described above.

[0015] The claimed invention including the methods described in the above embodiments is restricted to animals excluding humans.

[0016] The invention may find use in pathogens, such as *Salmonella*, transformed with a gene expressing a light-generating protein, such as luciferase.

[0017] The invention may find application in a method of identifying therapeutic compounds effective to inhibit spread of infection by a pathogen. The method may include administering a conjugate of the pathogen and a light-generating moiety to control and experimental animals, treating the experimental animals with a putative therapeutic compound, localizing the light-emitting pathogen in both control and experimental animals by the methods described above, and identifying the compound as therapeutic if the compound is effective to significantly inhibit the spread or replication of the pathogen in the experimental animals relative to control animals. The conjugates include a fluorescently-labeled antibodies, fluorescently-labeled particles, fluorescently-labeled small molecules, and the like.

[0018] In still another aspect, the invention includes a method of localizing entities conjugated to light-generating moieties through media of varying opacity. The method includes the use of photodetector device to detect photons transmitted through the medium, integrate the photons over time, and generate an image based on the integrated signal.

[0019] The invention may be useful for a method of measuring the concentration of selected substances, such as dissolved oxygen or calcium, at specific sites in an organism. The method may include entities, such as cells, containing a concentration sensor - a light-generating molecule whose ability to generate light is dependent on the concentration of the selected substance. The entity containing the light-generating molecule is administered such that it adopts a substantially uniform distribution in the animal or in a specific tissue or organ system (e.g. spleen). The organism is imaged, and the intensity and localization of light emission is correlated to the concentration and location of the selected substance. Alternatively, the entity contains a second marker, such as a molecule capable of generating light at a wavelength other than the concentration sensor. The second marker is used to normalize for any non-uniformities in the distribution of the entity in the host, and thus permit a more accurate determination of the concentration of the selected substance.

[0020] The invention may find use in a method of identifying therapeutic compounds effective to inhibit the growth and/or the metastatic spread of a tumor. The method includes (i) administering tumor cells labeled with or containing light-generating moieties to groups of experimental and control animals, (ii) treating the experimental group with a selected compound, (iii) localizing the tumor cells in animals from both groups by imaging photon emission from the light-generating molecules associated with the tumor cells with a photodetector device, and (iv) identifying a compound as therapeutic if the compound is able to significantly inhibit the growth and/or metastatic spread of the tumor in the experimental group relative to the control group.

[0021] The objects and features of the invention will be more fully appreciated when the following detailed description of the invention is read in conjunction with the accompanying reference examples and drawings.

BRIEF DESCRIPTION OF THE FIGURES

[0022] Figures 1A, 1B and 1C show a map of the *lux* pCGLS1 plasmid used to transform *Salmonella* strains SL1344, BJ66 and LB5000 to generate strains SL1344*lux*, BJ66*lux* and LB5000*lux*.

[0023] Figures 2A-E show the results of assays to measure adherence and invasion, by *Salmonella* strains SL1344*lux* and BJ66*lux*, of macrophages and HEp-2 cells. Figure 2A shows luminescent bacterial cells localized in wells of an assay dish. The pseudo-color image, obtained by integrating photons over one minute, is superimposed over a gray scale image of the assay dish, producing the "composite image" shown. Figure 2B shows the relative light intensity of wells that were not treated with gentamicin. Figure 2C shows the number of colony forming units (CFU) per ml isolated from the same wells as were imaged in Figure 2B. Figure 2D shows the relative light intensity of wells that were treated

with gentamicin. Figure 2E shows the number of colony forming units (CFU) per ml isolated from the same wells as were imaged in Figure 2D.

[0024] Figure 3A is a composite image of four glass capillary tubes containing dilutions of LB5000*lux* bacterial suspensions. Luminescence was determined by integrating over 30 seconds. Air pockets are present in each tube on both sides of the suspension.

[0025] Figure 3B depict the distribution of bioluminescence following intraperitoneal inoculation of wild-type SL1344*lux* into mice.

[0026] Figure 4 is a schematic diagram of a vial used to test the transmission of light generated by LB5000*lux* through animal tissue.

[0027] Figures 5A-F show composite images of Balb/c mice orally inoculated with low virulence LB5000*lux* (Figs. 5A-B), non-invasive BJ66*lux* (Figs. 5C-D) and virulent SL1344*lux* (Figs. 5E-F) *Salmonella*, and imaged at the times indicated in the figure. The luminescence signal was integrated over 5 minutes.

[0028] Figure 6 is a composite image showing the distribution of *Salmonella* in mice 32 hours following intraperitoneal (i.p.) injections with either virulent SL1344*lux* (left two animals) or low virulence LB5000*lux* (right two animals) strains of the bacterium.

[0029] Figures 7A and 7B show the distribution of virulent *Salmonella* in mice resistant to systemic *Salmonella* infections (129 × Balb/c, *Ity^{+/s}*). Figure 7A - day 1, Figure 7B - day 8.

[0030] Figures 8A-C show the distribution of mutant *Salmonella* with reduced virulence (BJ66*lux*) seven days following oral inoculation. Figure 8A shows external, non-invasive imaging of the luminescence. Figure 8B shows the same animal imaged following laparotomy. Labeled organs are C - cecum, L - liver, I - small intestine and Sp - spleen. Figure 8C shows a post-laparotomy image generated following injection of air into the lumen of the intestine both anterior and posterior to the cecum.

[0031] Figures 9A, 9B and 9C show the distribution of *Salmonella* SL1344*lux* in susceptible Balb/c mice following intraperitoneal inoculation with SL1344*lux*. Figure 9A was imaged prior to the opening of the peritoneal cavity. Figure 9B was imaged after the opening of the peritoneal cavity, and Figure 9C was imaged after the cecum was pulled to the left side.

[0032] Figures 10A-E show the effects of ciprofloxacin treatment on bioluminescence from SL1344*lux* *Salmonella* in orally-inoculated mice. Figure 10A shows a graph of the relative bioluminescence intensity, measured from the abdominal area, as a function of time after initiation of treatment, for treated and untreated animals. Figures 10B and 10D show composite images of mice 8 days after oral inoculation with SL1344*lux* *Salmonella*, before treatment with ciprofloxacin. Figures 10C and 10E show composite images of the same mice 5.5 hours either following treatment (Fig. 10E) or control (no treatment; Fig. 10C).

Definitions

[0033] Unless otherwise indicated, all terms used herein have the same meaning as they would to one skilled in the art of the present invention.

[0034] Opaque medium is used herein to refer to a medium that is "traditionally" opaque, not necessarily absolutely opaque. Accordingly, an opaque medium is defined as a medium that is commonly considered to be neither transparent nor translucent, and includes items such as a wood board, and flesh and skin of a mammal.

[0035] Luciferase, unless stated otherwise, includes prokaryotic and eukaryotic luciferases, as well as variants possessing varied or altered optical properties, such as luciferases that luminesce at wavelengths in the red range.

[0036] Biocompatible entity is an entity that can be administered to a mammal. This includes pathogens which may be deleterious to the mammal. In reference to an animal whose cells contain a transgene expressing a light-generating protein, biocompatible entity refers to the transgene-containing cells comprising the mammal.

[0037] Light-generating is defined as capable of generating light through a chemical reaction or through the absorption of radiation.

[0038] Light is defined herein, unless stated otherwise, as electromagnetic radiation having a wavelength of between about 300 nm and about 1100 nm.

[0039] Spread of infection typically refers to the spreading and colonization by a pathogen of host sites other than the initial infection site. The term can also include, however, growth in size and/or number of the pathogen at the initial infection site.

[0040] *lux* - prokaryotic genes associated with luciferase and photon emission.

[0041] *luc* - eukaryotic genes associated with luciferase and photon emission.

[0042] Promoter induction event refers to an event that results in the direct or indirect induction of a selected inducible promoter.

[0043] Heterologous gene refers to a gene which has been transfected into a host organism. Typically, a heterologous gene refers to a gene that is not originally derived from the transfected or transformed cells' genomic DNA.

General Overview of the Invention

[0044] The present invention includes methods and compositions relating to non-invasive imaging and/or detecting of light-emitting conjugates in mammalian subjects. The conjugates contain a biocompatible entity and a light-generating moiety. Biocompatible entities include, but are not limited to, small molecules such as cyclic organic molecules; macromolecules such as proteins; microorganisms such as viruses, bacteria, yeast and fungi; eukaryotic cells; all types of pathogens and pathogenic substances; and particles such as beads and liposomes. In another aspect, biocompatible entities may be all or some of the cells that constitute the mammalian subject being imaged.

[0045] Light-emitting capability is conferred on the entities by the conjugation of a light-generating moiety. Such moieties include fluorescent molecules, fluorescent proteins, enzymatic reactions giving off photons and luminescent substances, such as bioluminescent proteins. The conjugation may involve a chemical coupling step, genetic engineering of a fusion protein, or the transformation of a cell, microorganism or animal to express a bioluminescent protein. For example, in the case where the entities are the cells constituting the mammalian subject being imaged, the light-generating moiety may be a bioluminescent or fluorescent protein "conjugated" to the cells through localized, promoter-controlled expression from a vector construct introduced into the cells by having made a transgenic or chimeric animal.

[0046] Light-emitting conjugates are typically administered to a subject by any of a variety of methods, allowed to localize within the subject, and imaged. Since the imaging, or measuring photon emission from the subject, may last up to tens of minutes, the subject is usually, but not always, immobilized during the imaging process.

[0047] Imaging of the light-emitting entities involves the use of a photodetector capable of detecting extremely low levels of light - typically single photon events - and integrating photon emission until an image can be constructed. Examples of such sensitive photodetectors include devices that intensify the single photon events before the events are detected by a camera, and cameras (cooled, for example, with liquid nitrogen) that are capable of detecting single photons over the background noise inherent in a detection system.

[0048] Once a photon emission image is generated, it is typically superimposed on a "normal" reflected light image of the subject to provide a frame of reference for the source of the emitted photons (i.e. localize the light-emitting conjugates with respect to the subject). Such a "composite" image is then analyzed to determine the location and/or amount of a target in the subject.

[0049] The steps and embodiments outlined above are presented in greater detail, below.

Light-Emitting Entities

A. Light-Generating Moieties

[0050] The light-generating moieties (LGMs), molecules or constructs useful in the practice of the present invention may take any of a variety of forms, depending on the application. They share the characteristic that they are luminescent, that is, that they emit electromagnetic radiation in ultraviolet (UV), visible and/or infra-red (IR) from atoms or molecules as a result of the transition of an electronically excited state to a lower energy state, usually the ground state.

[0051] Examples of light-generating moieties include photoluminescent molecules, such as fluorescent molecules, chemiluminescent compounds, phosphorescent compounds, and bioluminescent compounds.

[0052] Two characteristics of LGMs that bear considerable relevance to the present invention are their size and their spectral properties. Both are discussed in the context of specific types of light-generating moieties described below, following a general discussion of spectral properties.

[0053] 1. Spectral Properties. An important aspect of the present invention is the selection of light-generating moieties that produce light capable of penetrating animal tissue such that it can be detected externally in a non-invasive manner. The ability of light to pass through a medium such as animal tissue (composed mostly of water) is determined primarily by the light's intensity and wavelength.

[0054] The more intense the light produced in a unit volume, the easier the light will be to detect. The intensity of light produced in a unit volume depends on the spectral characteristics of individual LGMs, discussed below, and on the concentration of those moieties in the unit volume. Accordingly, conjugation schemes that place a high concentration of LGMs in or on an entity (such as high-efficiency loading of a liposome or high-level expression of a bioluminescent protein in a cell) typically produce brighter light-emitting conjugates (LECs), which are easier to detect through deeper layers of tissue, than schemes which conjugate, for example, only a single LGM onto each entity.

[0055] A second factor governing the detectability of an LGM through a layer of tissue is the wavelength of the emitted light. Water may be used to approximate the absorption characteristics of animal tissue, since most tissues are composed primarily of water. It is well known that water transmits longer-wavelength light (in the red range) more readily than it does shorter wavelength light.

[0056] Accordingly, LGMs which emit light in the range of yellow to red (550 - 1100 nm) are typically preferable to LGMs which emit at shorter wavelengths. Several of the LGMs discussed below emit in this range. However, it will be

noted, based on experiments performed in support of the present invention and presented below, that excellent results can be achieved in practicing the present invention with LGMs that emit in the range of 486 nm, despite the fact that this is not an optimal emission wavelength. These results are possible, in part, due to the relatively high concentration of LGMs (luciferase molecules) present in the LECs (transformed *Salmonella* cells) used in these experiments, and to the use of a sensitive detector. It will be understood that through the use of LGMs with a more optimal emission wavelength, similar detection results can be obtained with LGEs having lower concentrations of the LGMs.

[0057] 2. Fluorescence-based Moieties. Fluorescence is the luminescence of a substance from a single electronically excited state, which is of very short duration after removal of the source of radiation. The wavelength of the emitted fluorescence light is longer than that of the exciting illumination (Stokes' Law), because part of the exciting light is converted into heat by the fluorescent molecule.

[0058] Because fluorescent molecules require input of light in order to luminesce, their use in the present invention may be more complicated than the use of bioluminescent molecules. Precautions are typically taken to shield the excitatory light so as not to contaminate the fluorescence photon signal being detected from the subject. Obvious precautions include the placement of an excitation filter, such that employed in fluorescence microscope, at the radiation source. An appropriately-selected excitation filter blocks the majority of photons having a wavelength similar to that of the photons emitted by the fluorescent moiety. Similarly a barrier filter is employed at the detector to screen out most of the photons having wavelengths other than that of the fluorescence photons. Filters such as those described above can be obtained from a variety of commercial sources, including Omega Optical, Inc. (Brattleboro, VT).

[0059] Alternatively, a laser producing high intensity light near the appropriate excitation wavelength, but not near the fluorescence emission wavelength, can be used to excite the fluorescent moieties. An x-y translation mechanism may be employed so that the laser can scan the subject, for example, as in a confocal microscope.

[0060] As an additional precaution, the radiation source can be placed behind the subject and shielded, such that the only radiation photons reaching the site of the detector are those that pass all the way through the subject. Furthermore, detectors may be selected that have a reduced sensitivity to wavelengths of light used to excite the fluorescent moiety.

[0061] Through judicious application of the precautions above, the detection of fluorescent LGMs according to methods of the present invention is possible.

[0062] Fluorescent moieties include small fluorescent molecules, such as fluorescein, as well as fluorescent proteins, such as green fluorescent protein (Chalfie, *et al.*, 1994, *Science* 263:802-805., Morin and Hastings, 1971, *J. Cell. Physiol.* 77:313) and lumazine and yellow fluorescent proteins (O'Kane, *et al.*, 1991, *PNAS* 88:1100-1104, Daubner, *et al.*, 1987, *PNAS* 84:8912-8916). In addition, certain colored proteins such as ferredoxin IV (Grabau, *et al.*, 1991, *J Biol Chem.* 266:3294-3299), whose fluorescence characteristics have not been evaluated, may be fluorescent and thus applicable for use with the present invention. Ferredoxin IV is a particularly promising candidate, as it has a reddish color, indicating that it may fluoresce or reflect at a relatively long wavelength and produce light that is effective at penetrating tissue. Furthermore, the molecule is small for a protein (95 amino acids), and can thus be conjugated to entities with a minimal impact on their function.

[0063] An advantage of small fluorescent molecules is that they are less likely to interfere with the bioactivity of the entity to which they are attached than would a larger light-generating moiety. In addition, commercially-available fluorescent molecules can be obtained with a variety of excitation and emission spectra that are suitable for use with the present invention. For example, Molecular Probes (Eugene, OR) sells a number of fluorophores, including Lucifer Yellow (abs. at 428 nm, and emits at 535 nm) and Nile Red (abs. at 551 nm and emits at 636 nm). Further, the molecules can be obtained derivatized with a variety of groups for use with various conjugation schemes (e.g. from Molecular Probes).

[0064] 3. Bioluminescence-based Moieties. The subjects of chemiluminescence (luminescence as a result of a chemical reaction) and bioluminescence (visible luminescence from living organisms) have, in many aspects, been thoroughly studied (e.g., Campbell, 1988, *Chemiluminescence. Principles and Applications in Biology and Medicine* (Chichester, England: Ellis Horwood Ltd. and VCH Verlagsgesellschaft mbH)). A brief summary of salient features follows.

[0065] Bioluminescent molecules are distinguished from fluorescent molecules in that they do not require the input of radiative energy to emit light. Rather, bioluminescent molecules utilize chemical energy, such as ATP, to produce light. An advantage of bioluminescent moieties, as opposed to fluorescent moieties, is that there is virtually no background in the signal. The only light detected is light that is produced by the exogenous bioluminescent moiety. In contrast, the light used to excite a fluorescent molecule often results in the fluorescence of substances other than the intended target. This is particularly true when the "background" is as complex as the internal environment of a living animal.

[0066] Several types of bioluminescent molecules are known. They include the luciferase family (e.g. Wood, *et al.*, 1989, *Science* 244:700-702) and the aequorin family (e.g. Prasher, *et al.*, *Biochem.* 26:1326-1332). Members of the luciferase family have been identified in a variety of prokaryotic and eukaryotic organisms. Luciferase and other enzymes involved in the prokaryotic luminescent (*lux*) systems, as well as the corresponding *lux* genes, have been isolated

from marine bacteria in the *Vibrio* and *Photobacterium* genera and from terrestrial bacteria in the *Xenorhabdus* genus.

[0067] An exemplary eukaryotic organism containing a luciferase system (*luc*) is the North American firefly *Photinus pyralis*. Firefly luciferase has been extensively studied, and is widely used in ATP assays. cDNAs encoding luciferases from *Pyrophorus plagiophthalmus*, another species of click beetle, have been cloned and expressed (Wood, *et al.*, 1989, *Science* 244:700-702). This beetle is unusual in that different members of the species emit bioluminescence of different colors. Four classes of clones, having 95-99% homology with each other, were isolated. They emit light at 546 nm (green), 560 nm (yellow-green), 578 nm (yellow) and 593 nm (orange). The last class (593 nm) may be particularly advantageous for use as a light-generating moiety with the present invention, because the emitted light has a wavelength that penetrates tissues more easily than shorter wavelength light.

[0068] Luciferases, as well as aequorin-like molecules, require a source of energy, such as ATP, NAD(P)H, and the like, and a substrate, such as luciferin or coelenterazine and oxygen.

[0069] The substrate luciferin must be supplied to the luciferase enzyme in order for it to luminesce. In those cases where a luciferase enzyme is introduced as an expression product of a vector containing cDNA encoding a *lux* luciferase, a convenient method for providing luciferin is to express not only the luciferase but also the biosynthetic enzymes for the synthesis of luciferin. In cells transformed with such a construct, oxygen is the only extrinsic requirement for bioluminescence. Such an approach, detailed in Example 1, is employed to generate *lux*-transformed *Salmonella*, which are used in experiments performed in support of the present invention and detailed herein.

[0070] The plasmid construct, encoding the *lux* operon obtained from the soil bacterium *Xenorhabdus luminescens* (Frackman, *et al.*, 1990, *J. Bact.* 172:5767-5773), confers on transformed *E. coli* the ability to emit photons through the expression of the two subunits of the heterodimeric luciferase and three accessory proteins (Frackman, *et al.*, 1990). Optimal bioluminescence for *E. coli* expressing the *lux* genes of *X. luminescens* is observed at 37°C (Sztitner and Meighen, 1990, *J. Biol. Chem.* 265:16581-16587, Xi, *et al.*, 1991, *J. Bact.* 173:1399-1405) in contrast to the low temperature optima of luciferases from eukaryotic and other prokaryotic luminescent organisms (Campbell, 1988, *Chemiluminescence. Principles and Applications in Biology and Medicine* (Chichester, England: Ellis Horwood Ltd. and VCH Verlagsgesellschaft mbH)). The luciferase from *X. luminescens*, therefore, is well-suited for use as a marker for studies in animals.

[0071] Luciferase vector constructs such as the one described above and in Example 1, can be adapted for use in transforming a variety of host cells, including most bacteria, and many eukaryotic cells (*luc* constructs). In addition, certain viruses, such as herpes virus and vaccinia virus, can be genetically-engineered to express luciferase. For example, Kovacs Sz. and Mettenlieter, 1991, *J. Gen. Virol.* 72:2999-3008, teach the stable expression of the gene encoding firefly luciferase in a herpes virus. Brasier and Ron, 1992, *Meth. in Enzymol.* 216:386-396, teach the use of luciferase gene constructs in mammalian cells. Luciferase expression from mammalian cells in culture has been studied using CCD imaging both macroscopically (Israel and Honigman, 1991, *Gene* 104:139-145) and microscopically (Hooper, *et al.*, 1990, *J. Biolum. and Chemilum.* 5:123-130).

B. Entities

[0072] The invention includes entities which have been modified or conjugated to include a light-generating moiety, construct or molecule, such as described above. Such conjugated or modified entities are referred to as light-emitting entities, light-emitting conjugates (LECs) or simply conjugates. The entities themselves may take the form of, for example, molecules, macromolecules, particles, microorganisms, or cells. The methods used to conjugate a light-generating moiety to an entity depend on the nature of the moiety and the entity. Exemplary conjugation methods are discussed in the context of the entities described below.

[0073] 1. Small molecules. Small molecule entities which may be useful in the practice of the present invention include compounds which specifically interact with a pathogen or an endogenous ligand or receptor. Examples of such molecules include, but are not limited to, drugs or therapeutic compounds; toxins, such as those present in the venoms of poisonous organisms, including certain species of spiders, snakes, scorpions, dinoflagellates, marine snails and bacteria; growth factors, such as NGF, PDGF, TGF and TNF; cytokines; and bioactive peptides.

[0074] The small molecules are preferably conjugated to light-generating moieties that interfere only minimally, if at all, with the bioactivity of the small molecule, such as small fluorescent molecules (described above). Conjugations are typically chemical in nature, and can be performed by any of a variety of methods known to those skilled in the art.

[0075] The small molecule entity may be synthesized to contain a light-generating moiety, so that no formal conjugation procedure is necessary. Alternatively, the small molecule entity may be synthesized with a reactive group that can react with the light generating moiety, or *vice versa*.

[0076] Small molecules conjugated to light-generating moieties of the present invention may be used either in animal models of human conditions or diseases, or directly in human subjects to be treated. For example, a small molecule which binds with high affinity to receptor expressed on tumor cells may be used in an animal model to localize and obtain size estimates of tumors, and to monitor changes in tumor growth or metastasis following treatment with a

putative therapeutic agent. Such molecules may also be used to monitor tumor characteristics, as described above, in cancer patients.

[0077] 2. Macromolecules. Macromolecules, such as polymers and biopolymers, constitute another example of entities useful in practicing the present invention. Exemplary macromolecules include antibodies, antibody fragments, fusion proteins and certain vector constructs.

[0078] Antibodies or antibody fragments, purchased from commercial sources or made by methods known in the art (Harlow, *et al.*, 1988, Antibodies: A Laboratory Manual, Chapter 10, pg. 402, Cold Spring Harbor Press), can be used to localize their antigen in a mammalian subject by conjugating the antibodies to a light-generating moiety, administering the conjugate to a subject by, for example, injection, allowing the conjugate to localize to the site of the antigen, and imaging the conjugate.

[0079] Antibodies and antibody fragments have several advantages for use as entities in the present invention. By their nature, they constitute their own targeting moieties. Further, their size makes them amenable to conjugation with several types of light-generating moieties, including small fluorescent molecules and fluorescent and bioluminescent proteins, yet allows them to diffuse rapidly relative to, for example, cells or liposomes.

[0080] The light-generating moieties can be conjugated directly to the antibodies or fragments, or indirectly by using, for example, a fluorescent secondary antibody. Direct conjugation can be accomplished by standard chemical coupling of, for example, a fluorophore to the antibody or antibody fragment, or through genetic engineering. Chimeras, or fusion proteins can be constructed which contain an antibody or antibody fragment coupled to a fluorescent or bioluminescent protein. For example, Casadei, *et al.*, 1990, *PNAS* 87:2047-2051, describe a method of making a vector construct capable of expressing a fusion protein of aequorin and an antibody gene in mammalian cells.

[0081] Conjugates containing antibodies can be used in a number of applications of the present invention. For example, a labeled antibody directed against E-selection, which is expressed at sites of inflammation, can be used to localize the inflammation and to monitor the effects of putative anti-inflammatory agents.

[0082] Vector constructs by themselves can also constitute macromolecular entities applicable to the present invention. For example, a eukaryotic expression vector can be constructed which contains a therapeutic gene and a gene encoding a light-generating molecule under the control of a selected promoter (i.e. a promoter which is expressed in the cells targeted by the therapeutic gene). Expression of the light-generating molecule, assayed using methods of the present invention, can be used to determine the location and level of expression of the therapeutic gene. This approach may be particularly useful in cases where the expression of the therapeutic gene has no immediate phenotype in the treated individual or animal model.

[0083] 3. Viruses. Another entity useful for certain aspects of the invention are viruses. As many viruses are pathogens which infect mammalian hosts, the viruses may be conjugated to a light-generating moiety and used to study the initial site and spread of infection. In addition, viruses labeled with a light-generating moiety may be used to screen for drugs which inhibit the infection or the spread of infection.

[0084] A virus may be labeled indirectly, either with an antibody conjugated to a light-generating moiety, or by, for example, biotinylating virions (e.g. by the method of Dhawan, *et al.*, 1991, *J. Immunol.* 147(1):102) and then exposing them to streptavidin linked to a detectable moiety, such as a fluorescent molecule.

[0085] Alternatively, virions may be labeled directly with a fluorophore like rhodamine, using, for example, the methods of Fan, *et al.*, 1992, *J. Clin. Micro.* 30(4):905. The virus can also be genetically engineered to express a light-generating protein. The genomes of certain viruses, such as herpes and vaccinia, are large enough to accommodate genes as large as the *lux* or *luc* genes used in experiments performed in support of the present invention.

[0086] Labeled virus can be used in animal models to localize and monitor the progression of infection, as well as to screen for drugs effective to inhibit the spread of infection. For example, while herpes virus infections can be manifested as skin lesions, this virus can also cause herpes encephalitis. Such an infection can be localized and monitored using a virus labeled by any of the methods described above, and various antiviral agents can be tested for efficacy in central nervous system (CNS) infections.

[0087] 4. Particles. Particles, including beads, liposomes and the like, constitute another entity useful in the practice of the present invention. Due to their larger size, particles may be conjugated with a larger number of light-generating molecules than, for example, can small molecules. This results in a higher concentration of light emission, which can be detected using shorter exposures or through thicker layers of tissue. In addition, liposomes can be constructed to contain an essentially pure targeting moiety, or ligand, such as an antigen or an antibody, on their surface. Further, the liposomes may be loaded with, for example, bioluminescent protein molecules, to relatively high concentrations (Campbell, 1988, Chemiluminescence. Principles and Applications in Biology and Medicine (Chichester, England: Ellis Horwood Ltd. and VCH Verlagsgesellschaft mbH)).

[0088] Furthermore, two types of liposomes may be targeted to the same cell type such that light is generated only when both are present. For example, one liposome may carry luciferase, while the other carries luciferin. The liposomes may carry targeting moieties, and the targeting moieties on the two liposomes may be the same or different. Viral proteins on infected cells can be used to identify infected tissues or organs. Cells of the immune system can be localized

using a single or multiple cell surface markers.

[0089] The liposomes are preferably surface-coated, e.g., by incorporation of phospholipid - polyethyleneglycol conjugates, to extend blood circulation time and allow for greater targeting via the bloodstream. Liposomes of this type are well known.

[0090] 5. Cells. Cells, both prokaryotic and eukaryotic, constitute another entity useful in the practice of the present invention. Like particles, cells can be loaded with relatively high concentrations of light-generating moieties, but have the advantage that the light-generating moieties can be provided by, for example, a heterologous genetic construct used to transfect the cells. In addition, cells can be selected that express "targeting moieties", or molecules effective to target them to desired locations within the subject. Alternatively, the cells can be transfected with a vector construct expressing an appropriate targeting moiety.

[0091] The cell type used depends on the application. For example, as is detailed below, bacterial cells, such as *Salmonella*, can be used to study the infective process, and to evaluate the effects of drugs or therapeutic agents on the infective process with a high level of temporal and spatial resolution.

[0092] Bacterial cells constitute effective entities. For example, they can be easily transfected to express a high levels of a light-generating moiety, as well as high levels of a targeting protein. In addition, it is possible to obtain *E. coli* libraries containing bacteria expressing surface-bound antibodies which can be screened to identify a colony expressing an antibody against a selected antigen (Stratagene, La Jolla, CA). Bacteria from this colony can then be transformed with a second plasmid containing a gene for a light-generating protein, and transformants can be utilized in the methods of the present invention, as described above, to localize the antigen in a mammalian host.

[0093] Pathogenic bacteria can be conjugated to a light-generating moiety and used in an animal model to follow the infection process *in vivo* and to evaluate potential anti-infective drugs, such as new antibiotics, for their efficacy in inhibiting the infection. An example of this application is illustrated by experiments performed in support of the present invention and detailed below.

[0094] Eukaryotic cells are also useful as entities in aspects of the present invention. Appropriate expression vectors, containing desired regulatory elements, are commercially available. The vectors can be used to generate constructs capable of expressing desired light-generating proteins in a variety of eukaryotic cells, including primary culture cells, somatic cells, lymphatic cells, etc. The cells can be used in transient expression studies, or, in the case of cell lines, can be selected for stable transformants.

[0095] Expression of the light-generating protein in transformed cells can be regulated using any of a variety of selected promoters. For example, if the cells are to be used as light-emitting entities targeted to a site in the subject by an expressed ligand or receptor, a constitutively-active promoter, such as the CMV or SV40 promoter may be used. Cells transformed with such a construct can also be used to assay for compounds that inhibit light generation, for example, by killing the cells.

[0096] Alternatively, the transformed cells may be administered such they become uniformly distributed in the subject, and express the light-generating protein only under certain conditions, such as upon infection by a virus or stimulation by a cytokine. Promoters that respond to factors associated with these and other stimuli are known in the art. In a related aspect, inducible promoters, such as the Tet system (Gossen and Bujard, 1992, *PNAS* 89:5547-5551) can be used to transiently activate expression of the light-generating protein.

[0097] For example, CD4+ lymphatic cells can be transformed with a construct containing tat-responsive HIV LTR elements, and used as an assay for infection by HIV (Israel and Honigman, 1991, *Gene* 104:139-145). Cells transformed with such a construct can be introduced into SCID-hu mice (McCune, *et al.*, 1988, *Science* 241:1632-1639) and used as model for human HIV infection and AIDS.

[0098] Tumor cell lines transformed as above, for example, with a constitutively-active promoter, may be used to monitor the growth and metastasis of tumors. Transformed tumor cells may be injected into an animal model, allowed to form a tumor mass, and the size and metastasis of the tumor mass monitored during treatment with putative growth or metastasis inhibitors.

[0099] Tumor cells may also be generated from cells transformed with constructs containing regulatable promoters, whose activity is sensitive to various infective agents, or to therapeutic compounds.

[0100] 6. Cell Transformation. Transformation methods for both prokaryotic cells and eukaryotic cells are well known in the art (Sambrook, *et al.*, 1989, In *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Vol. 2). Vectors containing the appropriate regulatory elements and multiple cloning sites are widely commercially available (e.g. Stratagene, La Jolla, CA, Clontech, Palo Alto, CA).

Transgenic Animals Containing Genes Encoding Light-Generating Proteins

[0101] In another aspect, the present invention includes transgenic animals containing a heterologous gene construct encoding a light-generating protein or complex of proteins. The construct is driven by a selected promoter, and can include, for example, various accessory proteins required for the functional expression of the light-generating protein,

as well as selection markers and enhancer elements.

[0102] Activation of the promoter results in increased expression of the genes encoding the light-generating molecules and accessory proteins. Activation of the promoter is achieved by the interaction of a selected biocompatible entity, or parts of the entity, with the promoter elements. If the activation occurs only in a part of the animal, only cells in that part will express the light-generating protein.

[0103] For example, an interferon-inducible promoter, such as the promoter for 3'-5' poly-A synthetase, can be used to detect the infection of transgenic cells by a number of different RNA viruses.

[0104] In a related aspect, a promoter expressed in certain disease states can be used to mark affected areas in a transgenic animal, and expression of the light-generating moiety can be used to monitor the effects of treatments for the disease state. For example, E-selection is expressed at sites of inflammation *in vivo* (Poher and Cotran, 1991, *Lab. Invest.* 64:301-305). Accordingly, the E-selection promoter can be isolated and used to drive the expression of a luciferase gene.

[0105] It is also possible to use methods of the invention with tissue-specific promoters. This enables, for example, the screening of compounds which are effective to inhibit pathogenic processes resulting in the degeneration of a particular organ or tissue in the body, and permits the tracking of cells (e.g. neurons) in, for example, a developing animal.

[0106] Many promoters which are applicable for use with the present invention are known in the art. In addition, methods are known for isolating promoters of cloned genes, using information from the gene's cDNA to isolate promoter-containing genomic DNA.

Imaging of Light-Emitting Conjugates

[0107] Light emitting conjugates that have localized to their intended sites in a subject may be imaged in a number of ways. Guidelines for such imaging, as well as specific examples, are described below.

A. Localization of Light-Emitting Conjugates

[0108] In the case of "targeted" entities, that is, entities which contain a targeting moiety - a molecule or feature designed to localize the entity within a subject or animal at a particular site or sites, localization refers to a state when an equilibrium between bound, "localized", and unbound, "free" entities within a subject has been essentially achieved. The rate at which such an equilibrium is achieved depends upon the route of administration. For example, a conjugate administered by intravenous injection to localize thrombi may achieve localization, or accumulation at the thrombi, within minutes of injection. On the other hand, a conjugate administered orally to localize an infection in the intestine may take hours to achieve localization.

[0109] Alternatively, localization may simply refer to the location of the entity within the subject or animal at selected time periods after the entity is administered. For example, in experiments detailed herein, *Salmonella* are administered (e.g., orally) and their spread is followed as a function of time. In this case, the entity can be "localized" immediately following the oral introduction, inasmuch as it marks the initial location of the administered bacteria, and its subsequent spread or recession (also "localization") may be followed by imaging.

[0110] In a related aspect, localization of, for example, injected tumors cells expressing a light-generating moiety, may consist of the cells colonizing a site within the animal and forming a tumor mass.

[0111] By way of another example, localization is achieved when an entity becomes distributed following administration. For example, in the case of a conjugate administered to measure the oxygen concentration in various organs throughout the subject or animal, the conjugate becomes "localized", or informative, when it has achieved an essentially steady-state of distribution in the subject or animal.

[0112] In all of the above cases, a reasonable estimate of the time to achieve localization may be made by one skilled in the art. Furthermore, the state of localization as a function of time may be followed by imaging the light-emitting conjugate according to the methods of the invention.

B. Photodetector Devices

[0113] An important aspect of the present invention is the selection of a photodetector device with a high enough sensitivity to enable the imaging of faint light from within a mammal in a reasonable amount of time, preferably less than about 30 minutes, and to use the signal from such a device to construct an image.

[0114] In cases where it is possible to use light-generating moieties which are extremely bright, and/or to detect light-emitting conjugates localized near the surface of the subject or animal being imaged, a pair of "night-vision" goggles or a standard high-sensitivity video camera, such as a Silicon Intensified Tube (SIT) camera (e.g. Hamamatsu Photonic Systems, Bridgewater, NJ), may be used. More typically, however, a more sensitive method of light detection is required.

[0115] In extremely low light levels, such as those encountered in the practice of the present invention, the photon flux per unit area becomes so low that the scene being imaged no longer appears continuous. Instead, it is represented by individual photons which are both temporally and spatially distinct from one another. Viewed on a monitor, such an image appears as scintillating points of light, each representing a single detected photon.

[0116] By accumulating these detected photons in a digital image processor over time, an image can be acquired and constructed. In contrast to conventional cameras where the signal at each image point is assigned an intensity value, in photon counting imaging the amplitude of the signal carries no significance. The objective is to simply detect the presence of a signal (photon) and to count the occurrence of the signal with respect to its position over time.

[0117] At least two types of photodetector devices, described below, can detect individual photons and generate a signal which can be analyzed by an image processor.

[0118] 1. Reduced-Noise Photodetection Devices. The first class constitutes devices which achieve sensitivity by reducing the background noise in the photon detector, as opposed to amplifying the photon signal. Noise is reduced primarily by cooling the detector array. The devices include charge coupled device (CCD) cameras referred to as "backthinned", cooled CCD cameras. In the more sensitive instruments, the cooling is achieved using, for example, liquid nitrogen, which brings the temperature of the CCD array to approximately -120°C. The "backthinned" refers to an ultra-thin backplate that reduces the path length that a photon follows to be detected, thereby increasing the quantum efficiency. A particularly sensitive backthinned cryogenic CCD camera is the "TECH 512", a series 200 camera available from Photometrics, Ltd. (Tucson, AZ).

[0119] 2. Photon Amplification Devices. A second class of sensitive photodetectors includes devices which amplify photons before they hit the detection screen. This class includes CCD cameras with intensifiers, such as microchannel intensifiers. A microchannel intensifier typically contains a metal array of channels perpendicular to and co-extensive with the detection screen of the camera. The microchannel array is placed between the sample, subject, or animal to be imaged, and the camera. Most of the photons entering the channels of the array contact a side of a channel before exiting. A voltage applied across the array results in the release of many electrons from each photon collision. The electrons from such a collision exit their channel of origin in a "shotgun" pattern, and are detected by the camera.

[0120] Even greater sensitivity can be achieved by placing intensifying microchannel arrays in series, so that electrons generated in the first stage in turn result in an amplified signal of electrons at the second stage. Increases in sensitivity, however, are achieved at the expense of spatial resolution, which decreases with each additional stage of amplification.

[0121] An exemplary microchannel intensifier-based single-photon detection device is the C2400 series, available from Hamamatsu.

[0122] 3. Image Processors. Signals generated by photodetector devices which count photons need to be processed by an image processor in order to construct an image which can be, for example, displayed on a monitor or printed on a video printer. Such image processors are typically sold as part of systems which include the sensitive photon-counting cameras described above, and accordingly, are available from the same sources (e.g. Photometrics, Ltd., and Hamamatsu). Image processors from other vendors can also be used, but more effort is generally required to achieve a functional system.

[0123] The image processors are usually connected to a personal computer, such as an IBM-compatible PC or an Apple Macintosh (Apple Computer, Cupertino, CA), which may or may not be included as part of a purchased imaging system. Once the images are in the form of digital files, they can be manipulated by a variety of image processing programs (such as "ADOBE PHOTOSHOP", Adobe Systems, Adobe Systems, Mt. View, CA) and printed.

C. Immobilizing Subject in Detection Field of Device

[0124] 1. Detection Field of Device. The detection field of the device is defined as the area from which consistent measurements of photon emission can be obtained. In the case of a camera using an optical lens, the detection field is simply the field of view accorded to the camera by the lens. Similarly, if the photodetector device is a pair of "night vision" goggles, the detection field is the field of view of the goggles.

[0125] Alternatively, the detection field may be a surface defined by the ends of fiber-optic cables arranged in a tightly-packed array. The array is constructed to maximize the area covered by the ends of the cables, as opposed to void space between cables, and placed in close proximity to the subject. For instance, a clear material such as plexiglass can be placed adjacent the subject, and the array fastened adjacent the clear material, opposite from the subject.

[0126] The fiber-optic cable ends opposite the array can be connected directly to the detection or intensifying device, such as the input end of a microchannel intensifier, eliminating the need for a lens.

[0127] An advantage of this method is that scattering and/or loss of photons is reduced by eliminating a large part of the air space between the subject and the detector, and/or by eliminating the lens. Even a high-transmission lens, such as the 60 mm AF Nikkor macro lens used in experiments performed in support of the present invention, transmits only a fraction of the light reaching the front lens element.

[0128] With higher-intensity LGMs, photodiode arrays may be used to measure photon emission. A photodiode array can be incorporated into a relatively flexible sheet, enabling the practitioner to partially "wrap" the array around the subject. This approach also minimizes photon loss, and in addition, provides a means of obtaining three-dimensional images of the bioluminescence.

[0129] Other approaches may be used to generate three-dimensional images, including multiple detectors placed around the subject or a scanning detector or detectors.

[0130] It will be understood that the entire animal or subject need not necessarily be in the detection field of the photodetection device. For example, if one is measuring a light-emitting conjugate known to be localized in a particular region of the subject, only light from that region, and a sufficient surrounding "dark" zone, need be measured to obtain the desired information.

[0131] 2. Immobilizing the Subject. In those cases where it is desired to generate a two-dimensional or three-dimensional image of the subject, the subject may be immobilized in the detection field of the photodetection devices during the period that photon emission is being measured. If the signal is sufficiently bright that an image can be constructed from photon emission measured in less than about 20 milliseconds, and the subject is not particularly agitated, no special immobilization precautions may be required, except to insure that the subject is in the field of the detection device at the start of the measuring period.

[0132] If, on the other hand, the photon emission measurement takes longer than about 20 msec, and the subject is agitated, precautions to insure immobilization of the subject during photon emission measurement, commensurate with the degree of agitation of the subject, need to be considered to preserve the spatial information in the constructed image. For example, in a case where the subject is a person and photon emission measurement time is on the order of a few seconds, the subject may simply be asked to remain as still as possible during photon emission measurement (imaging). On the other hand, if the subject is an animal, such as a mouse, the subject can be immobilized using, for example, an anesthetic or a mechanical restraining device.

[0133] A variety of restraining devices may be constructed. For example, a restraining device effective to immobilize a mouse for tens of seconds to minutes may be built by fastening a plexiglass sheet over a foam cushion. The cushion has an indentation for the animal's head at one end. The animal is placed under the plexiglass such that its head is over the indentation, allowing it to breathe freely, yet the movement of its body is constrained by the foam cushion.

[0134] In cases where it is desired to measure only the total amount of light emanating from a subject or animal, the subject does not necessarily need to be immobilized, even for long periods of photon emission measurements. All that is required is that the subject be confined to the detection field of the photodetector during imaging. It will be appreciated, however, that immobilizing the subject during such measuring may improve the consistency of results obtained, because the thickness of tissue through which detected photons pass will be more uniform from animal to animal.

D. Further Considerations During Imaging

[0135] 1. Fluorescent Light-Generating Moieties. The visualization of fluorescent light-generating moieties requires an excitation light source, as well as a photodetector. Furthermore, it will be understood that the excitation light source is turned on during the measuring of photon emission from the light-generating moiety.

[0136] Appropriate selection of a fluorophore, placement of the light source and selection and placement of filters, all of which facilitate the construction of an informative image, are discussed above, in the section on fluorescent light-generating moieties.

[0137] 2. High-Resolution Imaging. Photon scattering by tissue limits the resolution that can be obtained by imaging LGMs through a measurement of total photon emission. It will be understood that the present invention also includes embodiments in which the light-generation of LGMs is synchronized to an external source which can be focused at selected points within the subject, but which does not scatter significantly in tissue, allowing the construction of higher-resolution images. For example, a focused ultrasound signal can be used to scan, in three dimensions, the subject being imaged. Light-generation from areas which are in the focal point of the ultrasound can be resolved from other photon emission by a characteristic oscillation imparted to the light by the ultrasound (e.g. Houston and Moerner, U. S. Patent No. 4,614,116, issued 30 September 1986.)

E. Constructing an Image of Photon Emission

[0138] In cases where, due to an exceptionally bright light-generating moiety and/or localization of light-emitting conjugates near the surface of the subject, a pair of "night-vision" goggles or a high sensitivity video camera was used to obtain an image, the image is simply viewed or displayed on a video monitor. If desired, the signal from a video camera can be diverted through an image processor, which can store individual video frames in memory for analysis or printing, and/or can digitize the images for analysis and printing on a computer.

[0139] Alternatively, if a photon counting approach is used, the measurement of photon emission generates an array

of numbers, representing the number of photons detected at each pixel location, in the image processor. These numbers are used to generate an image, typically by normalizing the photon counts (either to a fixed, pre-selected value, or to the maximum number detected in any pixel) and converting the normalized number to a brightness (greyscale) or to a color (pseudocolor) that is displayed on a monitor. In a pseudocolor representation, typical color assignments are as follows. Pixels with zero photon counts are assigned black, low counts blue, and increasing counts colors of increasing wavelength, on up to red for the highest photon count values. The location of colors on the monitor represents the distribution of photon emission, and, accordingly, the location of light-emitting conjugates.

[0140] In order to provide a frame of reference for the conjugates, a greyscale image of the (still immobilized) subject from which photon emission was measured is typically constructed. Such an image may be constructed, for example, by opening a door to the imaging chamber, or box, in dim room light, and measuring reflected photons (typically for a fraction of the time it takes to measure photon emission). The greyscale image may be constructed either before measuring photon emission, or after.

[0141] The image of photon emission is typically superimposed on the greyscale image to produce a composite image of photon emission in relation to the subject.

[0142] If it desired to follow the localization and/or the signal from a light-emitting conjugate over time, for example, to record the effects of a treatment on the distribution and/or localization of a selected biocompatible moiety, the measurement of photon emission, or imaging can be repeated at selected time intervals to construct a series of images. The intervals can be as short as minutes, or as long as days or weeks.

Analysis of Photon Emission Images

[0143] Images generated by methods and/or using compositions of the present invention may be analyzed by a variety of methods. They range from a simple visual examination, mental evaluation and/or printing of a hardcopy, to sophisticated digital image analysis. Interpretation of the information obtained from an analysis depends on the phenomenon under observation and the entity being used.

[0144] The following experiments illustrate one application of the present invention - tracking *Salmonella* infection in live mice - and how images obtained using methods of the present invention can be analyzed.

Imaging of Luminescent *Salmonella* in Living Mice

[0145] Experiments performed in support of the present invention characterize the distribution of *Salmonella typhimurium* infection in mice, the animal model of human typhoid. A mouse virulent *Salmonella typhimurium* strain, SL1344 (Hoiseth and Stocker, 1981, *Nature* 291:238-239), a non-invasive mutant of SL1344, BJ66 and a low virulence LT-2 strain of *Salmonella*, LB5000 were each marked with a plasmid containing the *lux* operon, and used in experiments to localize *Salmonella* infection in mice.

A. Constructions of Luminescent *Salmonella*

[0146] 1. Salmonella Strains. Three strains of *Salmonella typhimurium* with differing virulence phenotypes, defined by oral and intra-peritoneal inoculations into mice, are selected for transformation.

[0147] The most virulent phenotype used herein is SL1344, a mouse strain originally obtained from a fatal infection of a calf (Hoiseth and Stocker, 1981, *Nature* 291:238-239). Following oral inoculations of mice with this strain, bacteria are disseminated systematically via the lymphatic system resulting in colonization of the liver, spleen and bone marrow (Carter and Collins, 1974, *J. Exper. Med.* 139:1189-1203.; see also reviews by Finlay and Falkow, 1989, *Mol. Microbiol.* 3:1833-1841, and Hsu, 1989, *Microbiol. Rev.* 53:390-409.)

[0148] A non-invasive mutant of SL1344, BJ66, is also evaluated. Systemic infections in mice do not typically result from an oral inoculation with BJ66, but do result from intraperitoneal inoculations with this strain.

[0149] A low virulence LT-2 strain of *Salmonella*, LB5000, is also examined. LT-2 strains are laboratory strains known to be of reduced or variable virulence for mice. LB5000 contains multiple auxotrophic mutations, is streptomycin resistant, and is cleared from mice following oral or intraperitoneal inoculations.

[0150] 2. Transformation of *Salmonella* Strains with the *lux* Operon. The three strains are each transformed with a plasmid encoding the *lux* operon, as detailed in Example 1. The plasmid, obtained from the soil bacterium *Xenorhabdus luminescens* (Frackman, *et al.*, 1990) confers on *E coli* the ability to emit photons through the expression of the two subunits of the heterodimeric luciferase and three accessory proteins, *luxC*, *luxD* and *luxE*.

[0151] Inclusion of *luxC*, *luxD* and *luxE* removes the necessity of providing the fatty aldehyde substrate, luciferin, to the luciferase-expressing cells. Because supplying the substrate to eukaryotic luciferase enzymes in an *in vivo* system such as described herein may prove difficult, the entire *lux* operon of *X. luminescens* is used. The operon also encodes the enzymes for the biosynthesis of the fatty aldehyde substrate.

[0152] *X. luminescens* luciferase, an alpha-beta heterodimeric mixed-function oxidase, catalyzes the oxidation of reduced flavin and long-chain aldehyde to oxidized flavin and the corresponding long-chain fatty acid. A fatty acid reductase complex is required for the generation and recycling of fatty acid to aldehyde, and an NAD(P)H:flavin oxidoreductase supplies the reduced flavin.

[0153] Optimal bioluminescence for *E. Coli* expressing the *lux* genes of *X. luminescens* is 37°C (Szittner and Meighen, 1990, *J. Biol. Chem.* 265:16581-16587, Xi, *et al.*, 1991, *J. Bact.* 173:1399-1405). In contrast, luciferases from eukaryotic and other prokaryotic luminescent organisms typically have lower temperature optima (Campbell, 1988, *Chemiluminescence. Principles and Applications in Biology and Medicine* (Chichester, England: Ellis Horwood Ltd. and VCH Verlagsgesellschaft mbH)). The luciferase from *X. luminescens*, therefore, is well-suited for use as a marker for studies in animals.

[0154] The three strains are transformed by electroporation with the plasmid pGSL1, which contains the entire *X. luminescens lux* operon and confers resistance to ampicillin and carbenicillin on the *Salmonella* (Frackman, *et al.*, 1990). The *X. luminescens lux* operon contains the genes *luxA*, *luxB*, *luxC*, *luxD* and *luxE* (Frackman, *et al.*, 1990). *LuxA* and *B* encode the two subunits of the heterodimeric luciferase. *luxC* and *D* encode the biosynthetic enzymes for the luciferase substrate and *luxE* is a regulatory gene. Inclusion of the genes for the biosynthesis of the substrate is a convenient means of providing substrate to luciferase, in contrast to supplying luciferin externally to the cells in culture or treating animals with the substrate.

B. Characterization of Transformed *Salmonella In Vitro*

[0155] 1. Adherence and Invasive Properties. The adherence and invasive properties of the three *Salmonella* strains containing the *lux* plasmid are compared in culture, to each other, and to their non-luminescent parental strains by the standard invasion assay as described by Finlay and Falkow, 1989, *Mol. Microbiol.* 3:1833-1841., and detailed in Example 2.

[0156] In this assay, adherent and intracellular bacteria are quantified following incubation with an epithelial cell line and peritoneal macrophages. The adherent and intracellular bacteria are detected and quantified by both the emission of photons from living cells, and colony forming units following lysis and plating the cell lysates on carbenicillin-containing plates.

[0157] The results of some of the assays are shown in Figures 2A through 2E and discussed in Example 8. The phenotypes of the three strains transformed with the *lux* expressing plasmid are not significantly altered in comparison to the parental *Salmonella* strains. In addition, there is a good correlation between the intensity of bioluminescence and the CFU from the HEp-2 cells and macrophages. The results show that luminescence, as an indicator of intracellular bacteria, is a rapid method for assaying the invasive properties of bacteria in culture.

[0158] BJ66 demonstrated reduced adherence to HEp-2 cells in comparison to SL1344, however, adherence of the two strains in primary cultures of murine peritoneal macrophages were comparable.

[0159] 2. Light Emission. To evaluate the oxygen requirements of the system, 10 fold serial dilutions of bacteria are placed in glass capillary tubes and imaged, as detailed in Example 3.

[0160] Figure 3 shows an image generated in one such experiment. Luminescence is only detected at the air-liquid interface, even in the tubes with small numbers of bacteria in air saturated medium (0.1 ml of air saturated buffer in 5 l results in a final O₂ concentration of 5 nM).

[0161] From these results, it is apparent that oxygen is likely a limiting factor for luminescence.

[0162] 3. Light Transmission Through Animal Tissue. To determine the degree to which light penetrates animal tissue, light emitted from luminescent *Salmonella* and transmitted through tissue is quantified using a scintillation counter, with the fast coincidence detector turned off to detect single photons. The background due to dark current of the photomultiplier tubes in this type of detection is significant, limiting the assay to samples with relatively strong photon emission.

[0163] Four tissue types of varying opacity are compared using this approach: muscle from chicken breast, skin from chicken breast, lamb kidney and renal medulla from lamb kidney. The number of photons that can be detected through tissue is approximately ten fold less than the controls without tissue.

4. Characterization of *lux Salmonella in vivo*.

[0164] a. Oral Administration. Oral inoculation is natural route of infection of mice or humans with *Salmonella* and results in a more protracted course of disease. In order to study the progression of the *Salmonella* infection following this route of inoculation, two strains of mice are infected with the three strains of *Salmonella*. The results obtained using the resistant animals are discussed under the heading "Infection of Resistant Mice", below.

[0165] Balb/c mice are orally infected with suspensions of virulent SL1344*lux*, non-invasive BJ66*lux* and low virulence LB5000*lux Salmonella*, as described in Example 5. Progression of the infection is followed by external imaging (Ma-

terials and Methods) over an 8 day period.

[0166] Representative images are shown in Figures 5A-F. At 24 hours post inoculation (p.i.), the bioluminescent signal is localized at a single focus in all infected animals (Figs. 5A, 5C and 5E). Bioluminescence disappears in all animals infected with the low virulence LB5000*lux* by 7 days p.i. (Fig. 5B). Animals infected with the virulent SL1344*lux*, on the other hand, show virulent infection which often spreads over much of the abdominal cavity (Fig. 5F), though the time at which it begins to spread is highly variable from animal to animal. The infection by BJ66*lux* typically persists and remains localized at a single site (Fig. 5D).

[0167] b. I.P. Inoculation. To assess whether or not there is sufficient O₂ at the sites of *Salmonella* replication for the oxidation of luciferin and subsequent luminescence (Campbell, 1988, *Chemiluminescence. Principles and Applications in Biology and Medicine* (Chichester, England: Ellis Horwood Ltd. and VCH Verlagsgesellschaft mbH)), photon emission is measured from the tissues of a respiring animal. Luminescent SL1344*lux* and LB5000*lux* are inoculated into the peritoneal cavities of two groups of Balb/c mice. 32 hours post inoculation (p.i.), the transmitted photons are imaged (Figure 6).

[0168] In the mice infected with SL1344*lux* (left part of figure), transmitted photons are evident over a large surface, with foci of varying intensities visible. These images are indicative of a disseminated infection, and are consistent with widespread colonization of the viscera, possibly including the liver and mesenteric lymph nodes. In contrast, the distributions of transmitted photons from animals infected with the LB5000*lux* strain is very limited, indicating a limited infection.

[0169] The LB5000*lux*-infected mice remained healthy for several weeks p.i., while the SL1344*lux*-infected mice were nearly moribund and euthanized at 4 days p.i.

[0170] These experiments indicate that the level of O₂ in the blood and or tissues is adequate for bioluminescence of lux luciferase expressed by *Salmonella*. Furthermore, the experiments are consistent with the invasive nature of the virulent strain SL1344 in comparison to the reduced virulent laboratory strain LB5000.

[0171] c. Infection of Resistant Mice. Mice which are heterozygous at the *Ity* locus (*Ity*^{r/s}) are resistant to systemic infections by *S. typhimurium* (Plant and Glynn, 1976, *J. Infect. Dis.* 133:72-78). This locus, also called *Bcg* (Gros, et al., 1981, *J Immunol.* 127:2417-2421) or *Lsh* (Bradley, 1977, *Clin. and Exper. Immunol.* 30:130-140), regulates the pathogenic processes of certain intracellular pathogens, such as *Mycobacterium lepraemurium* (Forget, et al., 1981, *Infect. Immunol.* 32:42-47), *M. Bovis* (Skamene, et al., 1984, *Immunogenet.* 19:117-120, Skamene and Pietrangeli, 1991, *Nature* 297:506-509) and *M. intracellulare* (Goto, et al., 1989, *Immunogenetics* 30:218-221). An analogous genetic control of resistance and susceptibility to intracellular pathogens appears to be in humans as well (*M. tuberculosis* (Stead, 1992, *Annals of Intern. Med.* 116:937-941, Stead, et al., et al., 1990, *New Eng. J. Med.* 322:422-427) and *M. leprae*).

[0172] The *Ity* locus is located on mouse chromosome 1 with two allelic forms, *Ity*^r (resistant, dominant) and *Ity*^s (sensitive, recessive). The gene encoded at the *Ity* locus apparently affects the ability of macrophages to disrupt the internalized pathogens (reviewed by Blackwell, et al., 1991, *Immunol. Lett.* 30:241-248 (1991); see also Skamene, et al., 1984, *Immunogenet.* 19:117-120, Skamene and Pietrangeli, 1991, *Nature* 297:506-509) which in turn, affects the down stream function of the proposed macrophage-mediated transport of pathogens to other sites within the infected host. Balb/c mice are *Ity*^{s/s} and 129 mice are *Ity*^{r/r}. The heterozygous Balb/c x 129 mice (*Ity*^{r/s}) are used in experiments detailed herein.

[0173] Resistant 129xBalb/c (*Ity*^{r/s}) viable mice are infected by intragastric inoculation of 1 × 10⁷ SL1344*lux Salmonella* as detailed in Example 7. The animals are imaged daily for 8 days post injection (d.p.i.).

[0174] Results are shown in Figures 7A (day 1) and 7B (day 8). The luminescence, detected by external imaging, is apparent at 24 h p.i., and appeared to be localized to a single site in all animals. The luminescent signal is present throughout the study period (up to 8 days p.i.). The intensity of the luminescence and the location of the luminescent source is somewhat variable over time within a mouse and also from mouse to mouse. The luminescent tissue in all infected animals is the cecum (see below) and the variability in localization, and possibly intensity, is most likely due to the fact that internal organs of rodents are not tightly fixed in position.

[0175] The apparent limited infection observed in these animals supports the interpretation that the *Ity* restriction blocks macrophage transport. The persistence of this infection for 10 days, however, suggests that there is adherence to the intestine mucosa and prolonged shedding of bacteria in the feces of these animals, as evidenced by luminescent fecal pellets. These results indicate that the luminescent phenotype of the *Salmonella* in vivo is retained over an 8 day duration in *Ity* restricted animals and that localization is possible following an oral inoculation.

[0176] d. Internal Imaging Following Oral Inoculation. In order to further localize the luminescent signal in the abdominal cavity, infected mice are imaged following laparotomy (Example 8). The predominant disease manifestation in all of the animals infected by the oral route is an enlarged cecum (Figs. 8A-C). The "external" image (Fig. 8A) illustrates a focal luminescence, which is revealed in the post-laparotomy image (Fig. 8B) to be the cecum.

[0177] Injection of air into the intestine confirms the presence of bacteria in other regions of the digestive tract. Bacteria in the colon and rectum are likely expressing luciferase, but low oxygen concentrations are likely limiting light

emission from these sites.

[0178] The images obtained from oral inoculation studies indicate that the luminescent signal, at 2 days p.i. and at 7 days p.i., localizes almost entirely to the cecum in each of the animals (Popesko, *et al.*, 1990, *A Colour Atlas of Anatomy of Small Laboratory Animals Vol. Two: Rat Mouse Hamster* (London England: Wolfe)) except those infected with LB5000*lux*. Luminescence is also apparent in the colon in some animals. By 7 days p.i., no luminescence is detectable in the LB5000*lux*-infected animals. The CFU present in the organs of these mice are determined at 2 and 5 d p.i.

[0179] In animals infected intragastrically with the invasive strain, SL1344*lux*, the luminescence in the cecum appears early and precedes a systemic infection. In contrast, infections with the non-invasive BJ66*lux* strain result in a persistent luminescence from the cecum that remains, in some animals, for the entire course of the study (8 days). By 8 days p.i., luminescence is detected over much of the abdominal surface, resembling the distribution of photons following an i.p. inoculation, in the SL1344*lux* infected mice.

[0180] Infections with SL1344*lux* appear to become systemic, as predicted, with progressively more photons being emitted from an increasing surface area. Luminescence appears to localize over the abdomen in infections with all strains with little detectable luminescence from outside this area. A large number of transmitted photons are localized as a single focus over the abdomen suggesting that even though the infection may be systemic, the greatest amount of replication may be in areas surrounding the intestine.

[0181] Localization of the luminescence over the cecum indicates that not only are there large numbers of organisms in this region of the intestine, but also suggests that the *Salmonella* associate with cells of the mucosa such that they can obtain sufficient oxygen for luminescence. Emission of photons from luciferase is oxygen dependent and the expected oxygen levels in the lumen of the cecum, or intestine in general, are below the levels required for luminescence. The luciferase reaction is not expected to be functional in the intestine unless the bacteria can obtain oxygen from cells of the intestinal epithelium.

[0182] Thus, the systemic infection seems to be related to the invasive phenotype and not to simply adherence to epithelial cells of the intestine. These experiments implicate the cecum in some role in the pathogenic process either in the carrier state or as a site of dissemination.

[0183] Monitoring the progression of infections to different tissues may greatly enhance the ability to understand these steps in the pathogenic process, and enable the screening for compounds effective to inhibit the pathogen at selected steps.

[0184] e. Internal Imaging Following I.P. Inoculation. Mice infected intraperitoneally with SL1344*lux* are imaged before and after laparotomy (Example 9). The results are shown in Fig. 9. The images demonstrate luminescence over a majority of the abdomen with multiple foci of transmitted photons. The cecum does not appear to contain luminescent *Salmonella*. The results from these experiments indicate that all strains of *Salmonella* have sufficient O₂ to be luminescent in the early phases of infection. However, entry of *Salmonella* into cells of the mucosa and subsequent systemic infection is likely limited to strains with the invasive phenotype, since systemic infections at later time points are only apparent in SL1344*lux*-infected mice.

[0185] f. Effects of Ciprofloxacin on *Salmonella* Infection. Experiments, detailed in Example 10, are performed to demonstrate that non-invasive imaging is useful for following the response of an infection to drugs. Mice are orally inoculated with SL1344*lux* and treated with 100 mg of ciprofloxacin, an antibiotic effective against *Salmonella* infections. The mice are imaged at selected time periods following treatment, and the extent of infection is quantitated by measuring photon emission. Photon emission in treated mice is compared to values before the initiation of treatment, and to values from control mice that had been infected, but not treated. Results from one such experiment are shown in Figures 10A-E and discussed in Example 10. Infection is significantly reduced in mice treated with the antibiotic, compared both to the levels of pathogen at time zero in treated animals, and to levels of pathogen in control animals throughout the treatment period.

[0186] g. Effects of Carbenicillin Selection. Ducluzeau, *et al.*, 1970, *Zeut. Bakt.* 5313:533-548., demonstrated that treatment of animals with antibiotics facilitated colonization of the cecum with *Salmonella*. The mice in the present experiments are maintained on an antibiotic regime of intramuscular injections of carbenicillin for the purpose of selecting the Amp^r *Salmonella* containing the luciferase clone. This treatment may alter the course of the gastrointestinal infection, but the observation that *Salmonella* can associate with the cells lining the cecum indicates that oxygen is available for luminescence. This observation is notable, since the lumen of the cecum is commonly thought to be an anaerobic environment.

Applications

[0187] The bioluminescence technology is broadly applicable to a variety of hostpathogen systems and may also enable temporal and spatial evaluation of other biological events, as for example tumor progression and gene expression in living mammals, and have application in pharmaceutical development and screening. Widespread use of *in*

vivo imaging of pathogens may reduce the numbers of animals and time needed for experiments pertaining to pathogenesis and/or the real-time study antimicrobial agents. Furthermore, bioluminescent organisms may be useful as biosensors in the living animal, much as luminescent bacteria are used in environmental analyses. Korpela *et al.*, for example, demonstrate that the limited oxygen supply in the lumen of the G.I. tract restricted bioluminescence to sites in which oxygen is accessible to the *Salmonella*, perhaps directly from epithelial or other cell types. Korpela, *et al.*, 1989, *J. Biolum. Chemilum.* 4:551-554. This oxygen requirement may find utility as an indicator of intimate cell-cell interactions, or as a biosensor for studying oxygen concentrations at various sites in living animals. In the following, several exemplary applications of this technology are described for the purpose of illustration, but are in no way intended to limit the present invention.

A. Determination of Oxygen Levels

[0188] The oxygen requirement for luminescence of luciferase evidenced in the experiments summarized above indicates that the present invention may be applicable as a method of determining spatial gradients of oxygen concentration in a subject. Luminescent bacteria have been used to measure oxygen levels in the range of 10 - 1 mM. The studies predict that 0.1 nM is the lower limit of detection (Campbell, 1988, *Chemiluminescence. Principles and Applications in Biology and Medicine* (Chichester, England: Ellis Horwood Ltd. and VCH Verlagsgesellschaft mbH)). The imaging methods described herein may be used for studying oxygen levels at various sites in living animals. For example, microorganisms that have been engineered to emit light in an O₂ or Ca²⁺-dependent manner could be used as biosensors in a subject, much like luminescent bacteria are used in environmental analyses (Guzzo, *et al.*, 1992, *Tox. Lett.* 64/65:687-693, Korpela, *et al.*, 1989, *J. Biolum. Chemilum.* 4:551-554, Jassim, *et al.*, 1990, *J. Biolum. Chemilum.* 5:115-122). The dynamic range of luminescence with respect to O₂ concentration is much broader and reaches lower O₂ concentrations than O₂ probes (Campbell, 1988, *Chemiluminescence. Principles and Applications in Biology and Medicine* (Chichester, England: Ellis Horwood Ltd. and VCH Verlagsgesellschaft mbH)). Moreover, light emission in proportion to O₂ concentration is linear over a range of 30 nM to 8 mM, and 9 mM O₂ is required for 1/2 maximal luminescence.

B. Localization of Tumor Cells

[0189] The growth and metastatic spread of tumors in a subject may be monitored using methods and compositions of the present invention. In particular, in cases where an individual is diagnosed with a primary tumor, LECs directed against the cells of the tumor can be used to both define the boundaries of the tumor, and to determine whether cells from the primary tumor mass have migrated and colonized distal sites.

[0190] For example, LECs, such as liposomes containing antibodies directed against tumor antigens and loaded with LGMs, can be administered to a subject, allowed to bind to tumor cells in the subject, imaged, and the areas of photon emission can be correlated with areas of tumor cells.

[0191] In a related aspect, images utilizing tumor-localizing LECs, such as those described above, may be generated at selected time intervals to monitor tumor growth, progression and metastasis in a subject over time. Such monitoring may be useful to record results of anti-tumor therapy, or as part of a screen of putative therapeutic compounds useful in inhibiting tumor growth or metastasis.

[0192] Alternatively, tumor cells can be transformed with a luciferase construct under the control of a constitutively-active promoter, and used to induce luminescent tumors in animal models, as described above. Such animal models can be used for evaluating the effects of putative anti-tumor compounds.

C. Localization of Inflammation

[0193] In an analogous manner to that described above, compositions and methods of the present invention may be used to localize sites of inflammation, monitor inflammation over time, and/or screen for effective anti-inflammatory compounds. Molecules useful for targeting to sites of inflammation include the ELAN family of proteins, which bind to selections. An ELAN molecule can be incorporated as a targeting moiety on an entity of the present invention, and used to target inflammation sites.

[0194] Alternatively, an animal model for the study of putative anti-inflammatory substances can be made by making the animal transgenic for luciferase under the control of the E-selection promoter. Since E-selection is expressed at sites of inflammation, transgenic cells at sites of inflammation would express luciferase.

[0195] The system can be used to screen for anti-inflammatory substances. Inflammatory stimuli can be administered to control and experimental animals, and the effects of putative anti-inflammatory compounds evaluated by their effects on induced luminescence in treated animals relative to control animals.

D. Localization of Infection

[0196] As illustrated in experiments performed in support of the present invention and summarized above, LGCs may be effectively used to follow the course of infection of a subject by a pathogen. In experiments detailed herein, the LGCs are pathogenic cells (*Salmonella*) transformed to express luciferase. Such a system is ideally-suited to the study of infection, and the subsequent spread of infection, in animal models of human diseases. It provides the ability to monitor the progression of an infectious disease using sites of infection and disease progression rather than traditional systemic symptoms, such as fever, swelling, etc. in studies of pathogenesis.

[0197] Use of an external imaging method to monitor the efficacy of anti-infectives permits temporal and spatial evaluations in individual living animals, thereby reducing the number of animals needed for experiments pertaining to pathogenesis and/or the study anti-infective agents.

[0198] The following examples illustrate, but in no way are intended to limit the present invention.

MATERIALS AND METHODS

A. Cells

[0199] *Salmonella* strains SL1344 and LB5000 were obtained from B.A.D. Stocker (Stanford University; Hoiseth and Stocker, 1981, *Nature* 291:238-239). *Salmonella* strain BJ66 was obtained from B.D. Jones (Stanford University).

[0200] HEP-2 cells were obtained from the American Type Culture Collection (ATCC; 12301 Parklawn Dr., Rockville MD; Accession number CCL-23).

[0201] Murine peritoneal macrophages were obtained by peritoneal lavage of euthanized Balb/c mice with 7 ml of growth medium (Maximow and Bloom, 1931, *Textbook of Histology*, Saunders, Philadelphia.)

B. Static Cultures

[0202] Low oxygen (static) cultures were prepared by inoculating 3 ml of LB Broth containing 100 mg/ml of carbenicillin with 6 μ l of a bacterial suspension from a stationary phase culture, and growing the bacteria at 37°C overnight in a stationary 7 ml culture tube.

C. Mice

[0203] Balb/c (*Ity^{s/s}*) mice were obtained from the Department of Oncology, Stanford University. 129xBalb/c (*Ity^{r/s}*) mice were obtained from the Stanford Transgenic Animal Facility (Stanford, CA). All animals were housed under identical conditions of photo period, feeding regime and temperature in the Stanford University Research Animal Facility (Stanford, CA).

[0204] Anesthesia was performed by injecting the animals intraperitoneally (i.p.) with 33 μ g/kg body weight nembutal.

[0205] Euthanasia was performed by asphyxiation in CO₂ or cervical dislocation, following protocols recommended by the Stanford University Research Animal Facility. Cervical dislocation was used in experiments in which results may have been affected by physiological changes due to asphyxia.

[0206] Mice infected with lux-transformed *Salmonella* were given daily intramuscular (i.m.) injections of carbenicillin (125 mg per kg body weight) to maintain selective pressure on the luminescent *Salmonella* for retention of the Amp^r plasmid containing the *lux* operon.

D. Imaging

[0207] Animals or objects to be imaged were immobilized in a light-tight box containing a door and a charge-coupled device (CCD) camera with a two stage microchannel intensifier head (model C2400-40, Hamamatsu). The camera was attached, via cables leading out of the box, to an "ARGUS 50" image processor (Hamamatsu).

[0208] The ICCD system described above is capable of detecting single photons once a threshold of 10-30 photons is achieved. The signal to noise ratio of the system ranged from 2:1 to 1x10⁴:1, depending on signal intensity.

[0209] Grey-scale images were obtained by opening the light box door in dim room light and integrating for 8 - 64 frames. The gain for the gray scale images was set to optimize the image - typically at 3000 volts on a scale of 0 to 10,000 volts.

[0210] Bioluminescence data were obtained in absence of external illumination. Exposure settings were as follows: the black level was set automatically by the camera/image processor, the gain was adjusted automatically by the intensifier controller, and the f-stop was set at 2.8. A 60 mm "AF NIKKOR" macro lens was used (Nikon Inc., Melville, NY).

[0211] Bioluminescence images were generated by integrating photons for a selected period of time, typically 5 minutes. Data are presented at the lowest bit range setting of 0-3 bits per pixel for all animals. For images of other objects, i.e. 24 well plates, where the resolution of the bioluminescent signals was not possible at a bit range of 0-3, the range was increased to a setting that permitted localization of bioluminescent signals, typically 1-7. Objects were imaged for shorter periods of time when additional information could not be obtained by imaging for five minutes.

[0212] External imaging refers to non-invasive imaging of animals. Internal imaging refers to imaging after a partial dissection of the animals, typically a laparotomy. Internal imaging is performed in selected animals to confirm the sources of photon emission localized by external imaging.

[0213] The bioluminescence image data are presented as a pseudo-color luminescence image representing the intensity of the detected photons. Six levels of intensity are typically used, ranging from blue (low intensity) to red (higher intensity).

[0214] To generate the figures presented herein, greyscale and bioluminescence images were superimposed, using the image processor, to form a composite image providing a spatial frame of reference.

[0215] The composite image was displayed on an RGB CRT (red, green, blue; cathode ray tube) monitor, and the monitor was photographed to produce hardcopies. Hardcopies were also generated by saving the image processor image as a digital file, transferring the file to a computer, and printing it on a color printer attached to the computer. Alternatively, hardcopies may be generated by printing the video signal directly using a video printer.

REFERENCE EXAMPLE 1

TRANSFORMATION OF *SALMONELLA* WITH pCGLS1 *LUX* PLASMID

[0216] *Salmonella* strains SL1344, BJ66 and LB5000 were transformed with pCGLS1, a pUC18-based vector encoding the *lux* operon from *Xenorhabdus luminescens* (Frackman, *et al.*, 1990).

A. pCGLS1 plasmid

[0217] A schematic of the pCGLS1 plasmid is shown in Figures 1A, 1B and 1C. The plasmid was constructed by cloning an ~11 kb region encoding the *lux* genes from the soil bacterium *Xenorhabdus luminescens* (Fig. 1A; Frackman, *et al.*, 1990) into the *Bam* HI site (Fig. 1B) of pUC18 (Fig. 1C; Clontech, Palo Alto, CA). The construction of the vector is described by Frackman, *et al.*, (1990).

[0218] Restriction enzyme sites in Figure 1A are represented as follows: Bs, *Bst* EII; C, *Cla* I; E, *Eco* RI; H, *Hind* III; M, *Mlu* I; S, *Sca* I; X, *Xba* I; B/Sa, *Bam* HI and *Sau* 3A junction. A sequence included in the multiple cloning site (MCS) is provided in Figure 1B, with the *Bam* HI site indicated in bold type.

[0219] A graphical representation of a pUC18 vector with no insert is shown in Figure 1C. Labeled elements include an ampicillin resistance gene (Ap), a *lac* Z gene (*lac* Z) and an *E. coli* origin of replication (*ori*). The unmodified pUC18 vector is approximately 2.7 kb in size.

B. Transformation of *Salmonella*

[0220] Electrocompetent cells from *Salmonella* strains SL1344, BJ66 and LB5000 were made using standard methods (Sambrook, *et al.*, 1989, In *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Vol. 2) and stored at -80°C until just prior to use. Electroporation was performed as follows: 1 µl of the plasmid (0.2 µg/ml) was added to 40 µl of ice-cold electrocompetent cells suspended in 10% glycerol. The suspension was mixed gently for one minute, placed in a 1 mm gap electroporation cuvette and electroporated using a Bio-Rad Gene-Pulser (Bio-Rad Laboratories, Hercules, CA). The settings were 2.5 kvolts, 400 ohms and 25 µfarads.

[0221] Following a one hour agitated incubation in Luria Bertini (LB) broth at 37°C, the cells were plated on (LB) Agar containing 100 µg/ml carbenicillin and allowed to grow overnight.

[0222] To maximize the bioluminescence of the labelled *Salmonella*, the *lux* operon was maintained on a high-copy-number plasmid and not integrated as a single copy gene. However, plasmids are subject to modification by the bacterial cell especially in *recA* strains, such as SL1344 and BJ66 used in this study. The *recA* locus encodes a recombinase that may delete regions of the plasmid containing the *lux* operon and the β-lactamase. Therefore, *Salmonella* recovered from cells in culture were plated both in the presence or absence of carbenicillin, and were imaged to determine the frequency at which bioluminescence was lost. All colonies recovered from gentamicin-treated, lysed HEP-2 cells and macrophages were ampicillin resistant (Amp^r) and bioluminescent. Therefore, *lux* genes appeared not to be lost during co-culture with mammalian cells.

[0223] Colonies were assayed for luminescence by visual inspection in a dark room. Five transformants were identified as having high levels of luminescence. Three of these, one each from the SL1344, BJ66 and LB5000 strains,

were selected for subsequent experiments. They were termed SL1344*lux*, BJ66*lux* and LB5000*lux*, respectively.

REFERENCE EXAMPLE 2

5 INVASIVE POTENTIAL OF NORMAL AND TRANSFORMED *SALMONELLA*

[0224] The invasive potential of six strains of *Salmonella* (SL1344*lux*, LB5000*lux*, BJ66*lux*, SL1344, LB5000 and BJ66) was determined using two types of bacterial adherence and entry assays. Colony-forming units (CFU) assays were performed essentially as previously described (Finlay and Falkow, 1989, *Mol. Microbiol.* 3:1833-1841) with modifications (Lee, *et al.*, 1990, *PNAS* 87:4304-4308). Bioluminescence assays were performed essentially like the CFU assays, except that the number of cells was quantitated using bioluminescence, as opposed to CFUs.

[0225] Briefly, HEP-2 cells and primary murine peritoneal macrophages were seeded into 24-well tissue culture dishes at 1×10^5 cells per well in RPMI (Gibco/BRL, Grand Island, NY) supplemented with 20 mM glutamine (Gibco/BRL) and 5% fetal calf serum (Hyclone, Logan, UT). Twenty four hours (HEP-2) or seven days (macrophages) after cell seeding, bacteria from static cultures (see "Materials and Methods", above) were inoculated at 1×10^6 (multiplicity of infection (m.o.i.) of 10) or 1×10^7 (m.o.i. of 100, columns on right in Figs. 2B-E) organisms per well and centrifuged onto the cell monolayer for 5 minutes at 1000 rpm ($185 \times g$) in a Beckman clinical centrifuge (Beckman Instruments, Columbia, MD). The medium was replaced with RPMI medium (Gibco/BRL) either with (entry assay) or without (adherence assay) gentamicin (100 mg/ml). The co-cultures were incubated for a total of 3.5 hours at 35°C in 5% CO₂.

[0226] Gentamicin in the incubation medium kills bacteria that had not been internalized by the HEP-2 cells, including those adhering to the surfaces of the HEP-2 cells. Accordingly, the signal in adherence assays (without gentamicin) represent both adherent and internalized bacteria, whereas the signal in entry assays (with gentamicin) represent only internalized bacteria.

[0227] Adherence and entry were assayed by imaging luminescent bacterial cells at three timepoints - 1.5, 3.0 and 3.5 hours post inoculation. Prior to imaging at the first timepoint, the cell monolayer was washed three times with phosphate-buffered saline (PBS) to remove unattached bacteria and a fresh aliquot of RPMI medium was added. Luminescence was recorded using a 30 second exposure. Images at the second and third timepoints were obtained using a similar exposure, but without first washing the cells.

[0228] Data recorded at the last timepoint, displayed as pseudocolor luminescence images superimposed over gray scale images of the culture dish wells, are shown in Figure 2A. The cell types, *Salmonella* strains, and usage of gentamicin are indicated in the Figure. The data are also summarized as relative intensity of photon counts in the graphs in Figures 2B and 2D.

[0229] Following imaging at the 3.5 hour timepoint, the tissue culture cells were washed three times with PBS and lysed with 0.2% "TRITON X-100" in PBS. Adherent and/or intracellular bacteria, released by lysis, were plated on LB- or LB-carbenicillin agar plates and incubated for 18 h at 35°C. The number of bacteria released from each well was determined by counting the number of colony forming units (CFU, Finlay and Falkow, 1989, *Mol. Microbiol.* 3: 1833-1841., Lee, *et al.*, 1990, *PNAS* 87:4304-4308). These data are represented as the total bacterial colonies per ml recovered from co-culture after incubation for 3.5 h with or without gentamicin, and are summarized in the graphs in Figures 2C and 2E.

[0230] Data from both the bioluminescence and CFU assays indicate that (i) *Salmonella* transformed with the *lux* genes have an infective potential similar to that of the parent lines, and (ii) luminescence detection and CFU determination yield comparable estimates for the invasive potential of the two *Salmonella* strains in HEP-2 cells and macrophages. The ratio of bioluminescence to CFU was lower in macrophage cultures, possibly due to the subcellular compartment in which the *Salmonella* enter macrophages.

45 REFERENCE EXAMPLE 3

IN VITRO LUMINESCENCE OF TRANSFORMED *SALMONELLA*

[0231] 10 μ l of four 10-fold serial dilutions (ranging from 10^6 cells to 10^3 cells per ml) of LB5000*lux* *Salmonella* were placed in four 100 μ l glass capillary tubes (Clay-Adams div. of Becton Dickinson, Parsippany, NJ). The bacterial suspensions formed columns of fluid in the tubes, with pockets of air at both ends. One end of each tube was sealed with critoseal (Clay-Adams). The medium in which dilutions were made was saturated with O₂ through exposure to air.

[0232] The tubes were wrapped with clear plastic wrap and luminescence was determined by imaging for 30 seconds as described above. An exemplary image is shown in Figure 3A. Four tubes are pictured. They contained (from top to bottom) 10^6 , 10^5 , 10^4 and 10^3 *Salmonella* cells/ml (10^4 , 10^3 , 10^2 and 10 cells/tube). Luminescence could be detected in suspensions containing as few as 10^4 cells/ml (100 cells). The luminescence is confined, however, to air/liquid interfaces, suggesting that the luminescence reaction requires relatively high levels of oxygen. Since many of the cells

are presumably in the fluid column and not at the air/fluid interfaces, the data suggest that the luminescence in the capillary tubes shown in Fig. 3A arises from considerably fewer than the total number of cells in each tube.

REFERENCE EXAMPLE 4

IN VITRO DETECTION OF LUMINESCENCE THROUGH ANIMAL TISSUE

[0233] Micro test-tubes, constructed from glass capillary tubing with an internal diameter of 3.5 mm, containing serial dilutions of LB5000/lux *Salmonella* were prepared essentially as described in Example 3, above. In the present example, however, the bacterial suspensions contacted the sealed end of the tube and were exposed to air only at the upper end. The tubes were placed in a translucent plastic scintillation vial and surrounded by one of the following animal tissues: chicken breast muscle, chicken skin, lamb kidney or lamb renal medulla. All tissues were obtained from the meat department of a local supermarket (Safeway, Mountain View, CA).

[0234] A diagram of a vial containing a capillary tube surrounded by tissue is shown in Figure 4. The vial 1 is approximately 1.4 cm in diameter and includes a cap 2. The vial is coated with an opaque material (i.e. black tape) along its upper portion 3. Animal tissue 4 is placed in the vial such that it extends from the bottom of the vial to just above the bottom edge of the opaque coating 3. The micro test-tube 5 is sealed at the bottom by a plug 7 (i.e. a cryoseal plug), and is centered radially in the vial, with the plugged end of the tube touching or in close proximity to the bottom of the vial. The bacterial suspension 6 extends approximately 1 cm upward from the bottom of the tube.

[0235] Photons emitted from vials with and without tissue, and with and without bacteria, were counted using a liquid scintillation counter (model 1219 Rackbeta, LKB/Wallac, Gaithersburg, MD) with the fast coincidence discriminator disabled.

[0236] Controls without tissue were assayed by placing the bacterial suspension directly in the scintillation vial. All experiments were performed in triplicate.

[0237] In each experiment, the vials were counted two to three times, rotating the vial 90° between each count, to control for effects of possible tissue thickness inconsistency. No significant differences were detected.

[0238] The results are summarized in Table 1, below.

TABLE 1

TRANSMISSION OF PHOTONS THROUGH TISSUE				
Sample	Chicken skin	Chicken muscle	Lamb kidney	Lamb medulla
Vial alone	2.1×10^4	1.3×10^4	1.0×10^4	1.0×10^4
Tissue alone	N.D.	1.5×10^4	9.4×10^3	8.5×10^3
Tissue and LB5000/lux*	2.7×10^5	2.3×10^5	1.6×10^4	1.5×10^5
LB5000/lux* alone	2.0×10^6	1.7×10^6	4.8×10^6	4.8×10^6

Counts are averages of triplicate measurements, tissue path length was 1 cm. * - 1×10^7 cells.

[0239] The signal for 1×10^3 LB5000/lux in kidney tissue was at or near background levels using the photomultiplier tubes (PMT) in the scintillation counter. The background in this type of detection is due to the dark current of the PMT and limits the studies to analysis of rather intense signals.

[0240] Bioluminescence from approximately 1×10^7 LB5000/lux was detectable through 0.5 cm of avian muscle, skin ovine renal medulla and ovine kidney. These results indicate that bioluminescence from the labeled *Salmonella* was detectable through animal tissues of variable opacity. Since oxygen was likely limited in the capillary tubes (as demonstrated in Fig. 3A), it is likely that fewer numbers of bioluminescent *Salmonella* could be detected through tissue than are indicated in this assay.

REFERENCE EXAMPLE 5

IN VIVO DETECTION OF BIOLUMINESCENT SALMONELLA

[0241] To assess the availability of oxygen to *Salmonella* during infection, wild-type SL1344lux was inoculated into the peritoneal cavity (i.p.) of BALB/c mice. Photons emitted from the bacteria internally, and transmitted through the abdominal wall were externally detected and localized in anaesthetized mice using an intensified CCD camera 24 h after inoculation (Fig. 3B). Systematic *Salmonella* infections are thought to involve colonization of the lymph nodes, spleen, liver, . Ventral images of the mice infected by i.p. inoculation of wild-type SL 1344lux demonstrated transmitted

photons over much of the abdominal surface, with foci of various intensities (Fig. 3B). These results were consistent with widespread colonization of the viscera, possibly including the liver and mesenteric lymph nodes, and indicate that the level of available oxygen in some tissues can be adequate for external detection of bioluminescence from the labelled pathogen.

Claims

1. A noninvasive method for detecting the integration of a transgene in a mammalian subject with the proviso that said mammalian subject is no human, comprising:

(a) administering to said subject a vector construct effective to integrate into mammalian cells and containing a transgene, a gene encoding a light-generating protein, and an activatable promoter, said gene encoding a light-generating protein being under the control of said promoter;

(b) after a period of time in which the construct can achieve integration, activating said promoter;

(c) placing the subject within the detection field of a photodetector device;

(d) maintaining the subject within the detection field of the photodetector device;

(e) during said maintaining, measuring the level of photon emission from expressed light-generating protein, in the subject, with the photodetector device; and

(f) confirming integration of the transgene if the level of photon emission is significantly higher than background.

2. A noninvasive method for detecting the localization of a promoter-induction event in an animal made transgenic for a construct including a gene encoding a light-generating protein under the control of an inducible promoter with the proviso that said animal is no human, comprising:

(a) triggering the event in a transgenic animal having an inducible promoter responsive to such event and a heterologous gene encoding a light-generating protein under control thereof;

(b) placing the animal in the detection field of a photodetector device;

(c) maintaining the animal in said photodetector device;

(d) during said maintaining, measuring photon emission from expressed light-generating protein, localized in the animal, with the photodetector device until an image of photon emission can be constructed; and

(e) constructing such an image.

3. The method of claim 2, wherein the animal is a mammal.

4. The method of claim 2, wherein the animal in step (c) is maintained in an immobilized condition.

5. The method of any one of claims 1 to 4, wherein the measuring is through opaque tissue.

6. The method of any one of claims 1 to 5, where the light-generating protein is luciferase, yellow fluorescent protein or ferredoxin IV.

7. The method of any one of claims 1 to 6, where said measuring is carried out with an intensified charge-coupled photodetector device.

Patentansprüche

1. Nicht-invasives Verfahren zum Nachweis der Integration eines Transgens in einen Säuger, wobei der Säuger kein Mensch ist, umfassend:

(a) Verabreichung eines Vektorkonstrukts an den Säuger, das in Säugerzellen integrieren kann und ein Transgen, ein ein Licht erzeugendes Protein codierendes Gen sowie einen aktivierbaren Promotor enthält, wobei das das Licht erzeugende Protein codierende Gen unter der Kontrolle des Promotors steht;

(b) Aktivieren des Promotors nach Ablauf einer Zeitspanne, in der das Konstrukt integrieren kann;

(c) Plazieren des Säugers in das Nachweisfeld einer Photodetektorvorrichtung;

(d) Halten des Säugers im Nachweisfeld der Photodetektorvorrichtung;

(e) während des Haltens, Messen des Photonenemissionsniveaus des exprimierten Licht erzeugenden Pro-

teins in dem Säuger mit der Photodetektorvorrichtung; und

(f) Bestätigen der Integration des Transgens, wenn das Photonenemissionsniveau signifikant höher als der Hintergrund ist.

- 5 2. Nicht-invasives Verfahren zum Nachweis der Lokalisation eines Promotorinduzierten Ereignisses in einem Tier, das mit einem Konstrukt transgen gemacht wurde, das ein ein Licht erzeugendes Protein codierendes Gen unter der Kontrolle eines induzierbaren Promotors beinhaltet, wobei das Tier kein Mensch ist, umfassend:
- 10 (a) Auslösen des Ereignisses in einem transgenen Tier, das einen induzierbaren Promotor, der auf ein solches Ereignis reagiert und ein heterologes Gen aufweist, das ein Licht erzeugendes Protein unter der Kontrolle des Promotors codiert;
- (b) Plazieren des Tiers in das Nachweisfeld einer Photodetektorvorrichtung;
- (c) Halten des Tiers in der Photodetektorvorrichtung;
- 15 (d) während des Haltens, Messen der Photonenemission des exprimierten Licht erzeugenden Proteins, das in dem Tier lokalisiert ist, mit der Photodetektorvorrichtung bis ein Bild der Photonenemission hergestellt werden kann; und
- (e) Herstellen eines solchen Bildes.
- 20 3. Verfahren nach Anspruch 2, wobei das Tier ein Säuger ist.
4. Verfahren nach Anspruch 2, wobei das Tier in Schritt (c) unter immobilisierenden Bedingungen gehalten wird.
5. Verfahren nach einem der Ansprüche 1 bis 4, wobei das Messen durch Licht undurchlässiges Gewebe geschieht.
- 25 6. Verfahren nach einem der Ansprüche 1 bis 5, wobei das Licht erzeugende Protein Luciferase, gelb-fluoreszierendes Protein ("yellow fluorescence protein") oder Ferredoxin IV ist.
7. Verfahren nach einem der Ansprüche 1 bis 6, wobei das Messen mit einer verstärkten Ladungs-gekoppelten Photodetektorvorrichtung ("intensified charge-coupled photodetector device") durchgeführt wird.
- 30

Revendications

- 35 1. Procédé non invasif pour détecter l'intégration d'un transgène chez un sujet mammifère, à la condition que ledit sujet ne soit pas humain, comprenant les étapes consistant à :
- (a) administrer audit sujet une construction de vecteur efficace à s'intégrer dans des cellules de mammifère et contenant un transgène, un gène codant pour une protéine luminescente et un promoteur pouvant être activé, ledit gène codant pour une protéine luminescente étant sous le contrôle dudit promoteur ;
- 40 (b) après une période pendant laquelle la construction peut accomplir son intégration, activer ledit promoteur ;
- (c) placer le sujet dans la portée du champ de détection du dispositif photodétecteur ;
- (d) maintenir le sujet dans la portée du champ de détection du dispositif photodétecteur ;
- (e) pendant ledit maintien, mesurer le niveau d'émission de photons par la protéine luminescente exprimée, chez le sujet, à l'aide du dispositif photodétecteur ; et
- 45 (f) confirmer l'intégration du transgène si le niveau d'émission de photons est significativement plus élevé que le bruit de fond.
2. Procédé non invasif pour détecter la localisation d'un événement d'induction de promoteur chez un animal rendu transgénique dans le cas d'une construction incluant un gène codant pour une protéine luminescente sous le contrôle d'un promoteur inductible, à la condition que ledit animal ne soit pas un être humain, comprenant les étapes consistant à :
- 50 (a) déclencher l'événement chez un animal transgénique porteur d'un promoteur inductible sensible à un tel événement et d'un gène hétérologue codant pour une protéine luminescente sous le contrôle de celui-ci ;
- (b) placer l'animal dans le champ de détection d'un dispositif photodétecteur ;
- 55 (c) maintenir l'animal dans ledit dispositif photodétecteur ;
- (d) pendant ledit maintien, mesurer l'émission de photons par ladite protéine luminescente exprimée, localisée chez l'animal, à l'aide du dispositif photodétecteur jusqu'à ce qu'une image d'émission de photons puisse être

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construite ; et
(e) construire une telle image.

3. Procédé selon la revendication 2, dans lequel l'animal est un mammifère.

5

4. Procédé selon la revendication 2, dans lequel l'animal dans l'étape (c) est maintenu dans un état immobilisé.

5. Procédé selon l'une quelconque des revendications 1 à 4, dans lequel on effectue la mesure à travers un tissu opaque.

10

6. Procédé selon l'une quelconque des revendications 1 à 5, où la protéine luminescente est la luciférase, la protéine fluorescente jaune ou la ferredoxine IV.

7. Procédé selon l'une quelconque des revendications 1 à 6, où l'on effectue ladite mesure à l'aide d'un dispositif photodétecteur à couplage de charge intensifié.

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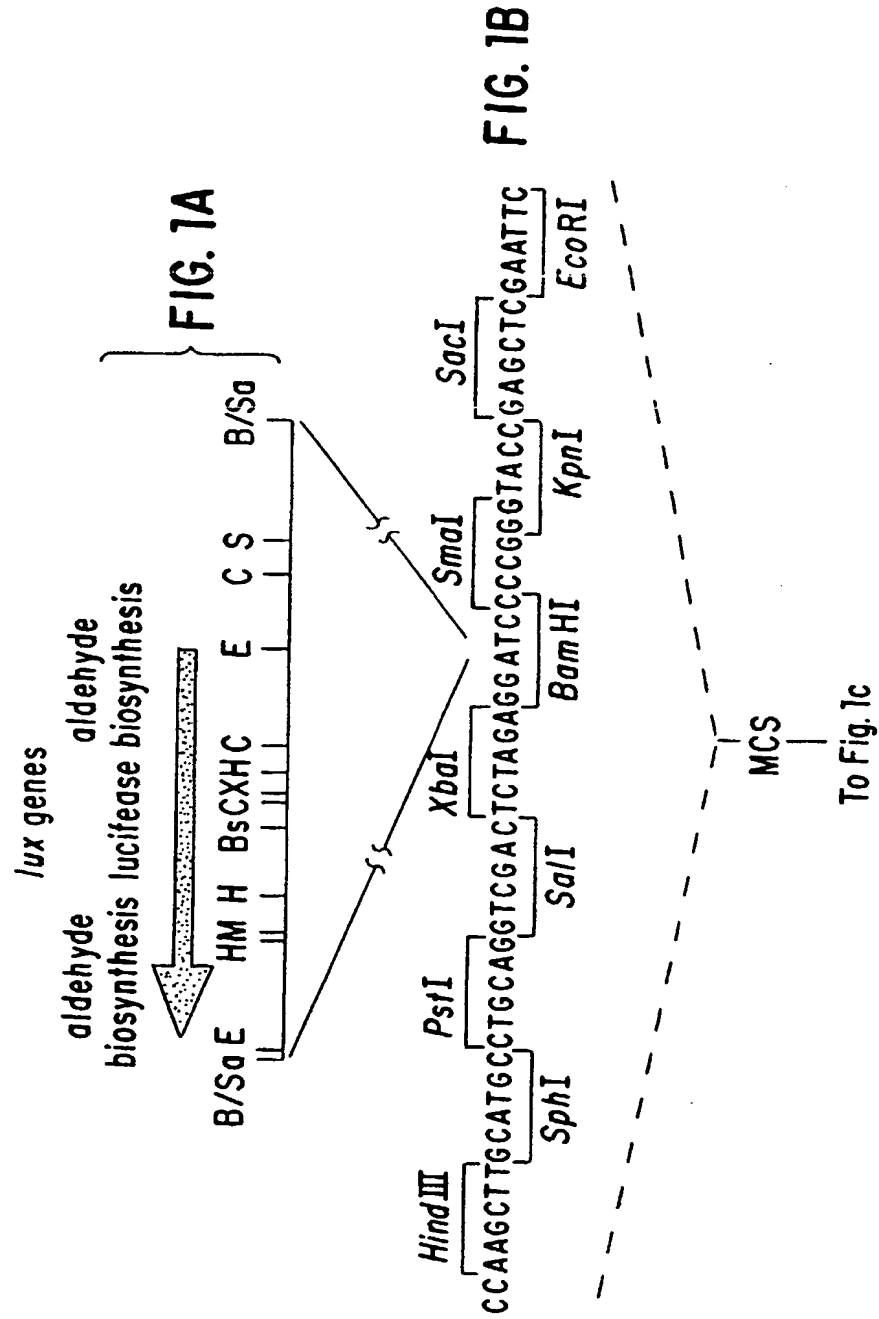
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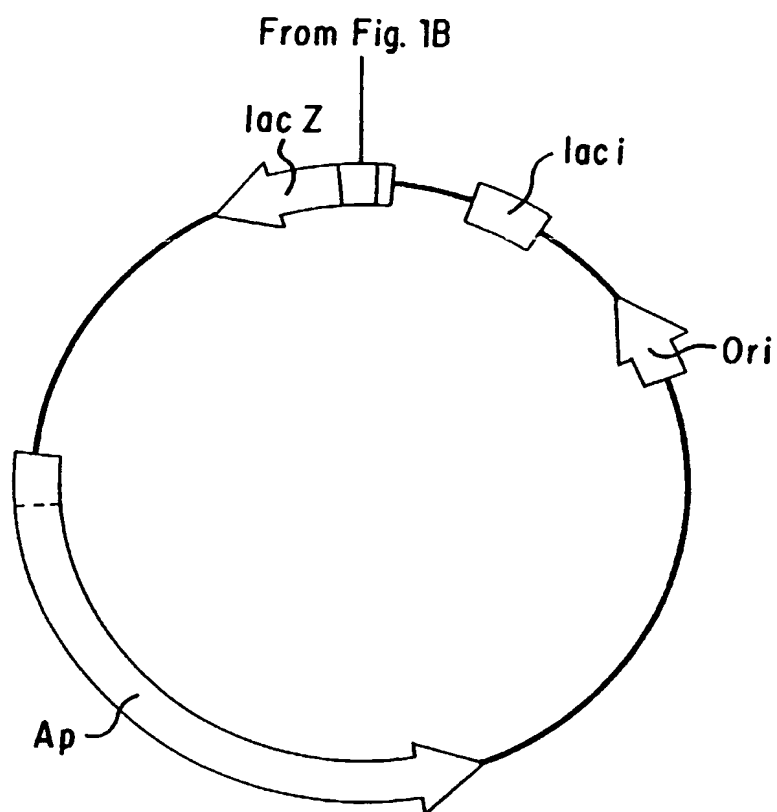


FIG. 1C



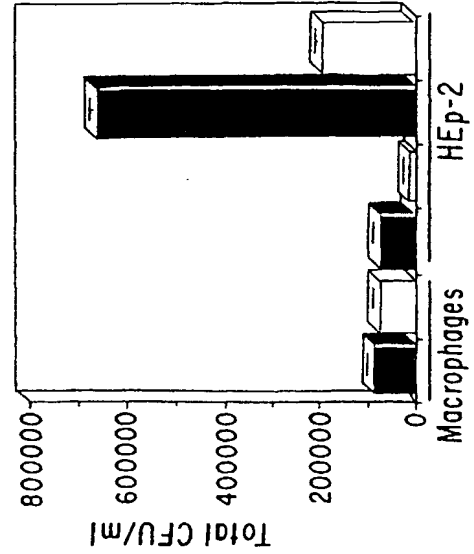
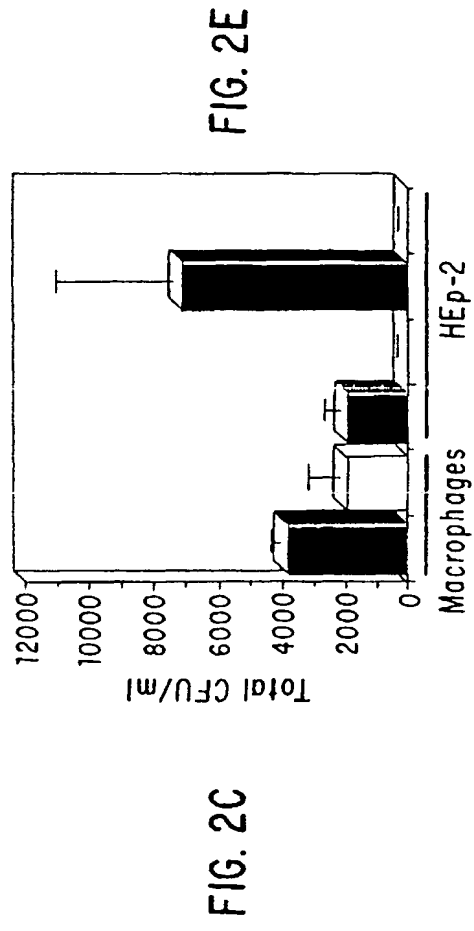
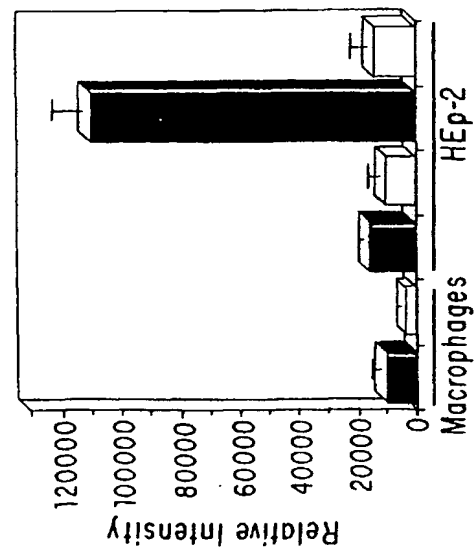
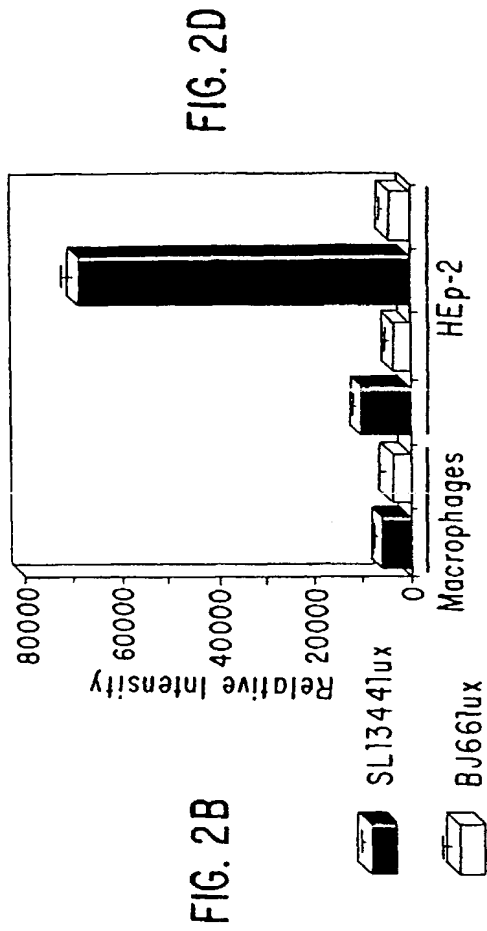




FIG.3A



1d

SL 1344lux
(intraperitoneal)

FIG.3B

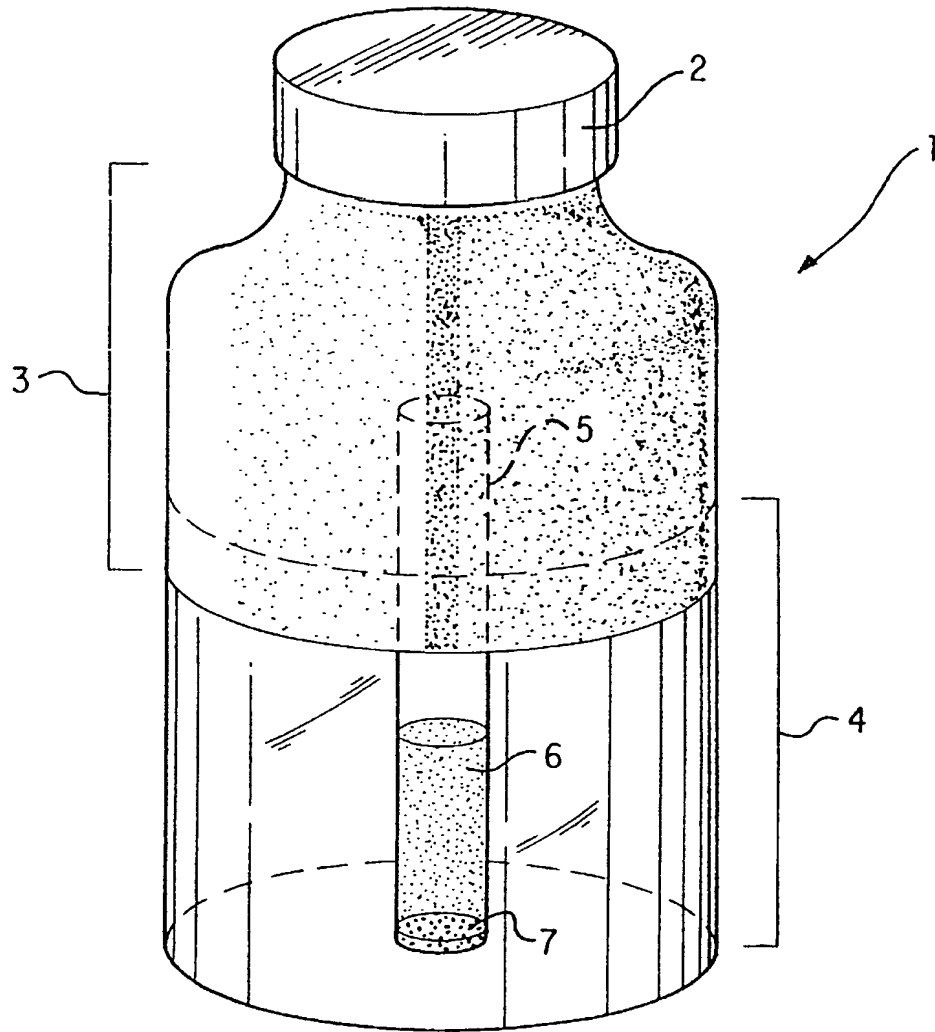


FIG. 4

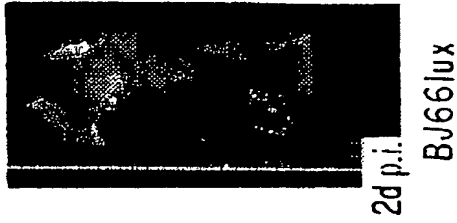


FIG. 5C

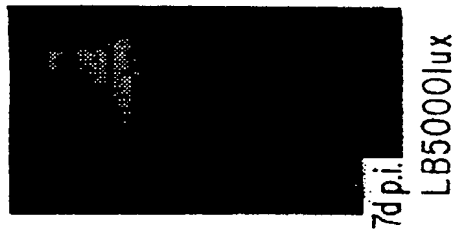


FIG. 5B



FIG. 5A



7d p.i.

BJ66lux

FIG. 5D



2d p.i.

SL1344lux

FIG. 5E



8d p.i.

SL1344lux

FIG. 5F



FIG.6

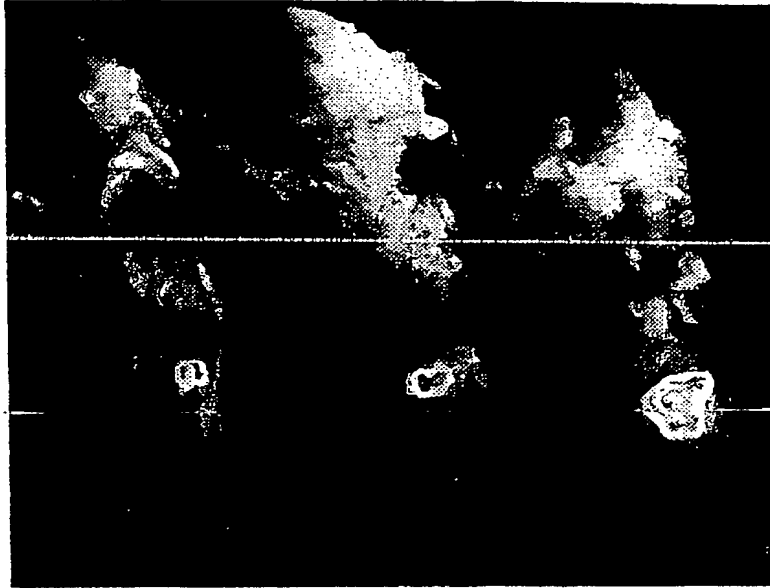


FIG.7A

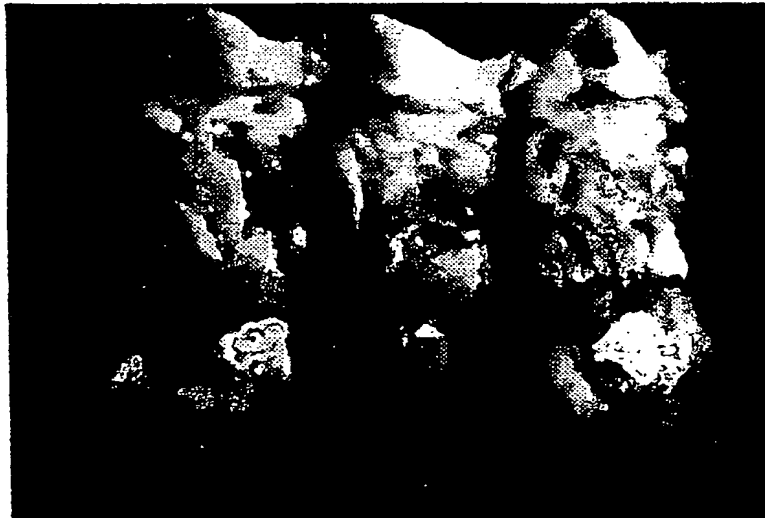
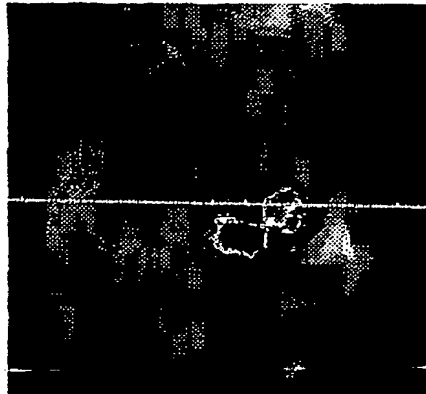
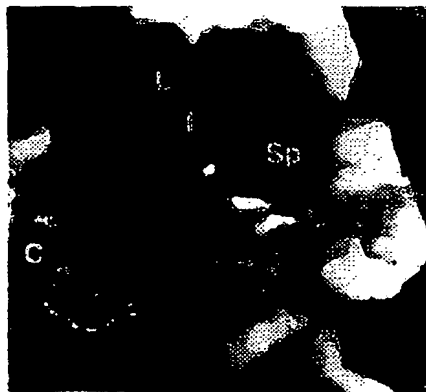


FIG.7B



BJ66lux Infection (7 d p.i.)

FIG.8A



BJ66lux Infection (7 d p.i.)

FIG.8B



BJ66lux Infection (7 d p.i.)

FIG.8C

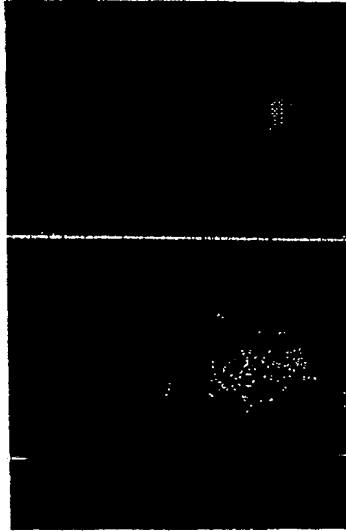


FIG. 9A

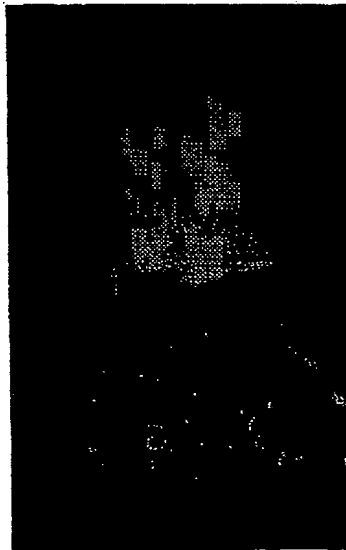


FIG. 9B

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FIG.9C

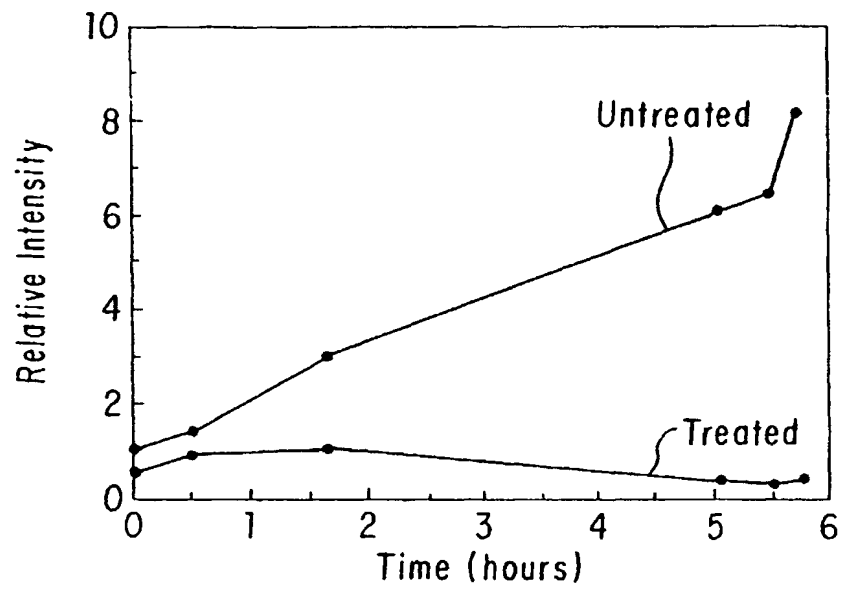


FIG. 10A

Untreated

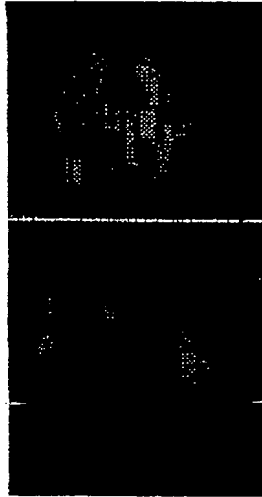


FIG.10B

Untreated



FIG.10C

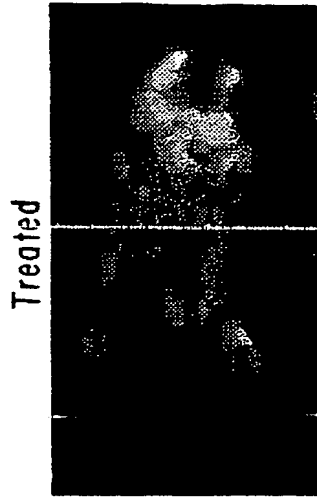


FIG.10D

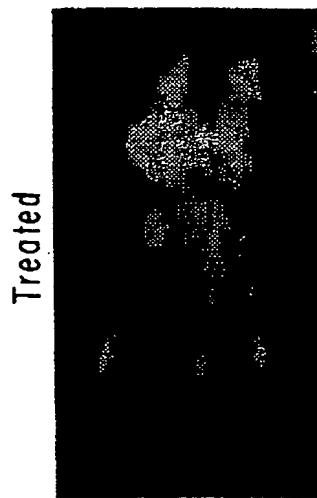


FIG.10E

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